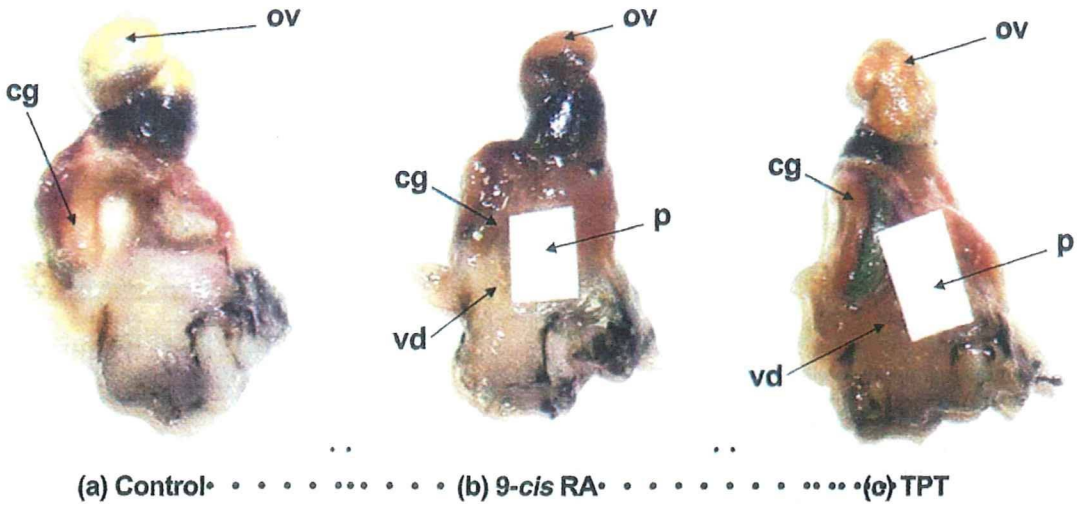

FIGURE 4. Substantial penis growth observed in the female rock shells after 1 month of 9-cis RA injections: cg, capsule gland; ov, ovary; p, penis; vd, vas deferens. (A) Neither penis nor vas deferens was observed in the control female (after shell removal). (B) Substantial penis growth as well as vas deferens development was observed in the female which received 9-cis RA injection (after shell removal; penis length: 6.06 mm). (C) Substantial penis growth as well as vas deferens development was also observed in the positive control female that received TPT injection (after shell removal; penis length: 6.50 mm). Imposex symptoms based on penis length and vas deferens sequence (VDS) index of the females that received 9-cis RA injections were clearly promoted, similar to those of females receiving TPT injections.

helix 4 in the ligand-binding domain (LBD) (*34*, *35*). By using degenerate primers deduced from these peptide sequences, we obtained a segment of *T. clavigera* RXR. Next, the *T. clavigera* cDNA library was screened to high precision using the RT-PCR product as a probe. Given that the cDNA isolated by screening was truncated, the 5′ end was amplified by RACE. Comparison of the rock shell RXR (sRXR) protein sequence with the Genebank database revealed that sRXR is closely related to vertebrate RXRs and invertebrate homologues (Figure 2). The highest homology with other species is in the DBD where 85−90% of the amino acids residues are identical (Figure 2). The LBD of sRXR also shows considerable homology with vertebrate RXRs but much less homology to ultraspiracle (USP), the RXR homologue found in *Drosophila*.

Ligand Binding Assay. Vertebrate RXRs bind to 9-cis RA, but insect USP does not (*30*, *31*, *36*). The LBD of sRXR protein, expressed after fusion with GST in bacteria, bound to 9-cis RA with $K_d = 15.2$ nM (Figure 3A,B), similar to values reported for vertebrate RXRs (*30*). These data implied that *T. clavigera* RXR could bind to 9-cis RA, even though *T. clavigera* is a gastropod mollusk. The sRXR fusion protein also bound to organotin compounds, such as TBT or TPT (Figure 3C). On the other hand, sRXR did not bind to all-trans RA (ATRA) in contrast to human RXRs that bind to ATRA even with low affinity (*30*) (Figure 3C; Figure 1E−G). The jellyfish RXR has also been reported to bind 9-cis RA with high affinity but not to ATRA (*37*).

In Vivo Injection Experiment To Examine the Involvement of RXR in the Development of Imposex in *Thais clavigera*: Effect of 9-cis RA Inducing and/or Promoting the Development of Imposex. To further verify the involvement of RXR in the development of imposex in gastropods, live female rock shells (*T. clavigera*) collected at Hiraiso in Ibaraki Prefecture, Japan (an area of low organotin contamination: see Horiguchi et al.; *18*) were injected with 9-cis RA. Results of these experiments are shown in Table 3 as well as Figure 4. Imposex was significantly induced in female *T. clavigera*, which received the injection of 9-cis RA ($p < 0.01$; Table 3), and substantial penis growth was observed in them after 1 month of 9-cis RA injections (Table 3; Figure 4). Their increased penis length and VDS index were significant when compared with controls ($p < 0.01$ and $p < 0.001$, respectively; Table 3).

TABLE 3. Incidence of Imposex (IOI), Penis Length (PL), and Vas Deferens Sequence Index (VDS) in Female Rock Shells (*Thais clavigera*) after 1 Month of Injections[a]

	control	RA	TPT
IOI (%)	10	50**	80**
PL (mm)	0.04 ± 0.13	2.87 ± 2.39**	3.77 ± 2.16***
VDS	0.20 ± 0.63	3.80 ± 0.42***	3.63 ± 0.74***

[a] Mean ± standard deviation. **, $p < 0.01$. ***, $p < 0.001$.

These results suggest that much 9-cis RA could bring about induction and/or promotion of the development of imposex in *T. clavigera* through its binding to RXR. Relatively large variance for the penis length in females that received injections of 9-cis RA may have resulted from differences in the rate of metabolism of 9-cis RA among female rock shells used in the experiment, although it is not known if *T. clavigera* inherently has a biosynthetic system for RA.

9-cis RA is the first substance, except for certain organotin compounds, that has been confirmed to induce and/or promote the development of imposex in gastopods, especially in terms of penis growth in females. As both TBT and TPT were observed to have agonistic activity to the RXR, it is strongly suggested that gastoropod imposex could be mediated by RXR.

Mode of Action of Organotins on the Development of Imposex in Gastropods. Several hypotheses have been proposed concerning the imposex induction mechanism, and they can be summarized as (i) increased androgen levels, such as testosterone, due to aromatase inhibition by TBT (*21*); (ii) inhibition by TBT of the excretion of sulfate conjugates of androgens (*22*); (iii) disturbance by TBT of penis morphogenic/retrogressive factor released from pedal/cerebropleural ganglia (*23*); and (iv) increase in a neuropeptide, APGWamide, level caused by TBT (*24*, *25*). Experimental evidence, however, is weak for these four hypotheses. There is a lack of correlation between the time course of the increase in testosterone titers and penis growth in females in the aromatase inhibition hypothesis (*21*), and there is a possibility that the results given in support of the testosterone excretion−inhibition hypothesis (*22*) may reflect a phenomenon that is at least partly short-term and/or associated with acutely

toxic TBT concentrations (*20*). The effect of APGWamide to induce and/or promote the development of imposex also appears weak based on experimental results of incidence of imposex and penis growth (*24, 25*).

In addition, it should be noted that substantial penis length has been observed in natural populations of imposex-exhibiting females distributed in coastal areas severely contaminated with TBT and/or TPT, as well as in females that received injections of or were exposed to TBT or TPT in the laboratory (*8–12, 16, 18*), and that little is known about basic endocrinology in invertebrates including mollusks (*38*). The penis length in female gastropods observed in the experiments given in support of the aromatase inhibition hypothesis, and the APGWamide involvement hypothesis was small (*21, 24, 25*). This contradiction concerning imposex development, especially penis length in imposex-exhibiting females, strongly suggested that gastropod imposex could be primarily induced and promoted by a factor other than increased androgen levels caused by aromatase inhibition or the neuropeptide, APGWamide. Moreover, there has not been any experimental evidence on purified aromatase protein itself (or aromatase at the protein level) in invertebrates, but only reports on aromatase-like activity in invertebrates including mollusks (*39–41*). The role of steroid sex hormones, similar to those of vertebrates, are still uncertain in invertebrates, because certain peptides have been reported to act as sex hormones in invertebrates such as *Aplysia californica* (Mollusca: Opisthobranchia), *Lymnaea stagnalis* (Mollusca: Plumonata), and *Armadillidium vulgare* (Arthropoda: Malacostraca) (*42–44*). In contrast, RXR is rather well-conserved from invertebrates to vertebrates (Figure 2).

In this paper, we have shown that TBT and TPT are high affinity ligands for RXR and that the natural ligand of RXR significantly caused the development of imposex in female rock shells. These results imply that RXR plays an important role in the induction/differentiation and growth of male genital tracts in female gastropods. Further studies on a heterodimer partner, coupling factors, and target genes of sRXR with molecular biological and immunohistochemical techniques are necessary to clarify the entire mode of action of TBT and/or TPT on the development of imposex in gastropods.

RXRs are key factors involved in the mediation of several hormone response systems via their association with other nuclear receptors as heterodimer partner (*45*). The knockouts of RXRs in the mouse have provided important information in the physiological functions of these receptors. RXRα null mice died in utero and exhibited a hypoplasic ventricular myocardium and ocular abnormalities (*46, 47*). Approximately 50% of RXRβ null mice died before or at birth, and males of the remaining null mutants were sterile, owing to the aberrant lipid metabolism in Sertoli cells (*48*). On the other hand, 9-cis RA is difficult to detect in vivo, and its action is remained to be obscure (*49*). Our result that injection of 9-cis RA into female gastropods induced and/or promoted the development of imposex may provide some insight into the physiological function of 9-cis RA.

Acknowledgments

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Original

Observation of Preputial Separation is a Useful Tool for Evaluating Endocrine Active Chemicals

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Abstract: Flutamide, *p,p'*-dichlorodiphenyldichloroethylene, vinclozolin, diethylstilbestrol, ethynylestradiol and tamoxifen were administered by gavage to pregnant Sprague-Dawley rats on gestational days 14–17 or 18–21, and to male offspring on postnatal days 1–5, 17–21 or 35–39. The influence on the sexual maturation was assessed by preputial separation. Cleft phallus with hypospadias was induced by prenatal exposure to 10 mg/kg flutamide on gestational days 14–17 and 18–21, or administration of 100 mg/kg vinclozolin on gestational days 14–17 to the dams. The day of preputial separation in these offspring could not be determined, because complete separation did not occur. Prenatal exposure of males to other chemicals did not affect the preputial separation. Postnatal exposure of 10 and 30 mg/kg flutamide and 30 mg/kg vinclozolin led to delays of preputial separation. A marked delay was observed in males exposed to 100 μg/kg of ethynylestradiol or 3 mg/kg of tamoxifen on postnatal days 1–5. Diethylstilbestrol, 300 μg/kg, administration on postnatal days 1–5 and 35–39 caused a delay in preputial separation. These results indicate that observing preputial separation is useful for evaluating anti-androgen treatment in the prepubertal period, and estrogen-related chemical treatment from the neonatal period. (J Toxicol Pathol 2005; **18**: 141–157)

Key words: preputial separation, rat, flutamide, vinclozolin, ethynylestradiol, diethylstilbestrol

Introduction

Preputial separation, which is the separation of the prepuce from the glans penis, is used as an indication of puberty in the male rat. Histological observations on the progress of preputial separation after cornification at the lining of the prepuce and surface of the glans penis were well described using Long-Evans rats in 1942[1]. We showed similar histological changes in Sprague-Dawley rats examined from postnatal day (PND) 6 to PND 56[2]. Preputial separation is thought to be dependent on androgens, since castration blocks preputial separation and the addition of testosterone (TS) or dihydrotestosterone (DHT) nullifies the effect of castration[1,3]. In recent years, various *in vivo* screening assays have been developed for detecting endocrine disrupting chemicals. The uterotrophic assay is a method for detecting estrogenic or anti-estrogenic effects of chemicals on the weight of the uterus using immature or ovariectomized female rats. The Hershberger assay is a screening test to detect androgenic or anti-androgenic effects of chemicals on the weight of castrated male reproductive organs such as the ventral prostate and seminal vesicles. The enhanced OECD Test Guideline 407 is a draft, new version of the Repeated Dose 28-day Oral Toxicity Study in Rodents, and is designed to detect the endocrine effect of chemicals. Also, the rodent 20-day thyroid/pubertal male assay has been proposed for evaluating chemicals influencing male puberty. In this assay, weaning male rats are continuously dosed by gavage beginning one week before puberty (which occurs at about PND 40) until PND 53, and their puberty is measured by determining their age at preputial separation. Both estrogenic and anti-androgenic chemicals may induce delays in male puberty. Although the observation of preputial separation is a useful tool for detecting sexual maturation, the adequate administration period or relationship between dose and effect has not been sufficiently investigated. Accordingly, we performed a preliminary study to find out the suitable administration period or the most sensitive period using the following well-known chemicals: flutamide, *p,p'*-dichlorodiphenyldichloroethylene, vinclozolin, diethylstilbestrol, ethynylestradiol and tamoxifen. Flutamide (FLU)[4,5] is an anti-androgenic drug and is used in the Hershberger assay as the positive control agent. *p,p'*-dichlorodiphenyldichloroethylene (DDE)[4–7] and vinclozolin (VZ)[7,8] are anti-androgenic chemicals and are used in the OECD validation study of the Hershberger assay to verify

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the effectiveness of this screening assay. Diethylstilbestrol (DES) is an estrogenic compound and has been used in various experimental studies. Ethynylestradiol (EE) is a synthetic estrogen and is used as a positive control in the uterotrophic assay, and tamoxifen (TAM) is used as a positive control for the anti-estrogenic effect in this screening assay.

To determine the administration period, the following reports were used as references. Induction of hypospadias has been reportedly caused by anti-androgens[9]. Puberty is undetermined in males with hypospadias because complete separation in the glans penis is not evident in these animals. A sensitive prenatal period for hypospadias is known to exist, thus, we selected gestational days (GD) 18–21 as the low sensitive period for prenatal exposure. We selected GD 14–17 as the high sensitive period for comparative study, but the high dose group of some chemicals may not be used for the observation of preputial separation. To examine the effects on the neonatal period, newborn rats were orally administered test chemicals on PND 1–5. Leydig cells of neonatal rats are known as fetal Leydig cells, and adult type Leydig cells appear from about PND 14[10]. It was reported that male rat serum testosterone levels reach a maximum on GD 19, decrease on PND 12, and then increase[11]. Thus, we also chose to administer the test chemicals during PND 17–21 and PND 35–39 (prepubertal period). The 4 or 5 days of administration period in the present study is short compared to the Hershberger assay (10 days) or pubertal male assay (20 days), thus the effect on the animals is thought to be less than that seen in these assays. The purpose of this preliminary study was to find out the sensitive period for the screening assay, and more prolonged administration may show more pronounced effects on the animals. The number of animals in each group was determined referring to the other screening assays such as the uterotrophic assay (6 female rats in a group), the Hershberger assay (6 male rats in a group), and the Enhanced OECD Test Guideline 407 or pubertal male assay (10 male rats and 10 female rats). The number of pups and litters may be insufficient to determine the effects of unknown chemicals, but the purpose of the present study was to obtain the suitable period of administration for a screening study by the observation of preputial separation, thus we performed the preliminary study using the experimental design described in the following section.

Materials and Methods

Animals and housing conditions

Sprague-Dawley rats (Crj:CD (SD) IGS), 210 males and 421 females, 11 weeks of age, were obtained from Charles River Japan, Inc. (Atsugi, Japan). All animals were acclimatized to laboratory conditions and quarantined for about one week before mating. Rats used for this study were selected based upon their general appearance and behavior during the acclimatization period. Animals were housed individually in wire-bottom metal cages (220 × 270 × 190 mm) and kept in a barrier sustained animal room that was maintained at 21.0 – 25.0°C and 40.0 – 75.0% relative humidity with a 12-hour artificial light cycle (lighting from 7:00 to 19:00). The room air was changed fifteen times per hour, and a commercial diet, CE-2 (CLEA Japan, Inc., Tokyo, Japan), and water (Hadano City) were available *ad libitum* throughout the study. The Animal Use Committee of the Hatano Research Institute approved the study protocol.

To obtain pregnant animals, 12-week-old females were cohabited overnight on a 1:1 basis with males 12 weeks of age or older. Females were considered to be at GD 0 when daily examination revealed a vaginal plug. All pregnant animals were housed in cages with animal bedding, PAPER CLEAN® (Japan SLC, Inc., Shizuoka, Japan), from GD 18 and allowed to give birth. The dams and pups were housed in wire-bottom metal cages after postpartum day 10.

For prenatal exposure, pregnant females were randomly assigned to groups consisting of 3 to 5 based on body weight before administration. For PND 1–5 exposure (PND 0 is the day of delivery), all female pups were discarded on PND 1 and the number of males per litter was adjusted to 5. The litters were then allocated to groups consisting of 3 or 4 litters (except for one group consisting 2 litters) based on pup mean body weight. On PND 6, the number of pups for prenatal or neonatal exposure and that for premature exposure was adjusted to 4 males per litter. For PND 17–21 and 35–39 exposure, litters were allocated to groups a few days before administration based on mean body weight. The numbers of litters or pups were decided referring to the other screening assays.

Chemicals and treatment

FLU, DES, EE and TAM were purchased from Sigma-Aldrich (St. Louis, MO), DDE was obtained from Aldrich Chemical (Milwaukee, WI), and VZ from Wako Pure Chemical (Osaka, Japan). Each chemical was dissolved in corn oil (Nacalai Tesque, Inc., Kyoto, Japan). Dosage levels for FLU were 1, 10 and 30 mg/kg/day, for DDE 10, 30, 100 and 300 mg/kg/day, for VZ 10, 30 and 100 mg/kg/day, for DES 0.1, 1, 10, 100 and 300 μg/kg/day, for EE 10 and 100 μg/kg/day, and for TAM 0.03 and 0.1 mg/kg/day (prenatal exposure) or 0.3, 1 and 3 mg/kg/day (postnatal exposure). The dosages employed in this study were based on the results from preliminary studies or those reported in the literature.

The chemicals were orally administered by gavage (5 or 10 mL/kg BW) to pregnant female rats on GD 14–17 or 18–21, or to males on PND 1–5, 17–21 or 35–39 (Fig. 1). In addition, EE was administered on PND 6–10 or 11–15. The control animals were administered vehicle corn oil orally, at the same time periods and volume as the test group. An ATOM indwelling feeding tube (Atom Medical, Tokyo, Japan) was used for neonatal administration as described by Watanabe[12] and a stomach tube was used for adult and premature animals.

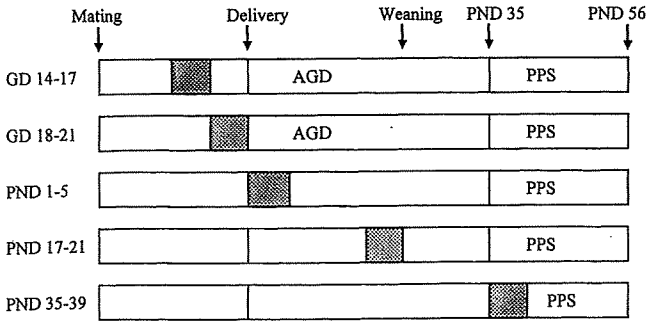

Fig. 1. Schedule of prenatal and postnatal treatment.
▨: period of chemical exposure, GD: gestational day, PND: postnatal day, AGD: measurement of anogenital distance on PND 6, PPS: observation of preputial separation from PND 35.

Examination and measurement

Body weights of pregnant females were measured on GD 0, on the day of grouping before administration, and during the administration period. Body weights of males were measured on PND 0, 6, 22, 35, 56, the day of complete preputial separation, on the day of grouping, and during the administration period. Body weights on PND 0 to 6 were measured as mean value of each litter, and after PND 22 they were measured individually. The anogenital distance (AGD) of prenatally exposed male pups was measured on PND 6 before adjusting to 4 males per litter, with a digital micrometer (reproducible precision of 0.01 mm, Digimatic caliper CD-15CP, Mitutoyo Corporation, Kanagawa, Japan). The progress of preputial separation in the males was observed macroscopically[13] from PND 35. Surviving males were sacrificed under anesthesia on PND 56 and autopsy was performed. Male fatalities were autopsied as early as possible after they were discovered. Following the macroscopic examination, the testes, epididymides, ventral prostate, seminal vesicles, prepuce and penis were excised and fixed in 0.1 mol/L phosphate buffered 10% formalin solution. Weights of the testes, epididymides, ventral prostate, and seminal vesicles of sacrificed animals were measured on the next day. Representative organs of 169 cases were embedded in paraffin, and the sections were then stained with hematoxylin-eosin (H&E) for histopathological examination. To observe early stage hypospadias some of the discarded males were sacrificed on PND 6 and examined histologically as described above.

Statistical analysis

Body weights on the day of preputial separation and autopsy, the day of preputial separation, organ weights, AGD, and correction values of AGD [AGD (mm)/ $\sqrt[3]{\text{body weight (g)}}$ of pups] were statistically analyzed using the litter as the unit. Body weights on the day of preputial separation, the day of preputial separation, and organ weights in postnatally administered males were also analyzed using the individual values. These data were analyzed using Bartlett's test. When homogeneity of variance was confirmed, one-way analysis of variance was applied to detect significant differences among the groups. If a significant difference was detected among the groups, Dunnett's test was applied for multiple comparisons. When variance was not homogeneous, the Kruskal-Wallis analysis of ranks was applied. If a significant effect was detected among the groups, Dunnett's test was applied for multiple comparisons. The day of preputial separation and the body weights on the day of preputial separation between the two groups were analyzed by Student's or Welch's t-test. Comparisons between groups were made using $P \leq 0.05$ as the level of significance. When preputial separation was not complete on the day of autopsy, the day of separation was set as day 56 for the analysis. The correlation between the day of preputial separation and body weight was analyzed using Pearson's correlation coefficient.

Results

Prenatal exposure

1) Effects on the pregnant females

In the groups exposed to 300 mg/kg of DDE on GD 14–17 or GD 18–21, body weights decreased during the administration period and either the pregnant females or their pups died. Depression of the weight gain was also observed in the 100 mg/kg DDE group exposed on GD 18–21, and all the pups in 3 litters out of 4 died. Deaths of pregnant females or pups were observed in the VZ, DES and TAM groups. Although depression of weight gain was observed in the EE group, all females survived and delivered pups. In the VZ, DES and TAM groups, one or two females delivered before the last day of administration period, and these females were excluded from the experiment.

2) Malformations and inflammatory lesions of the genital organ

The incidence of malformations and inflammatory lesions after prenatal exposure to chemicals are summarized in Table 1. On macroscopic examination, the glans penis of control males was covered with prepuce, and the prepuce could be completely retracted to expose the glans penis before PND 56 (Figs. 2A, 2B). The prepuce of males from dams exposed to 10 mg/kg of FLU on GD 14–17 had a cleft at the ventral aspect (cleft prepuce) and the glans penis was observed from the cleft. The ventral part of the penis was incompletely formed (cleft phallus) and the os penis was often exposed. The external urethral orifice of males with a cleft phallus opened at the ventral surface of the penis (hypospadias). Hypospadias was usually observed with cleft phallus. The incidence of a cleft prepuce was 25% (3 cases in 2 litters) and the incidence of a cleft phallus was 58% (7 cases in all of the 3 litters), although a cleft prepuce was usually observed with a cleft phallus. Five males had no cleft on their prepuce or phallus. Although there was no cleft at the prepuce of males from dams exposed to FLU on GD 18–21, the ventral part of the penis was incompletely formed (cleft phallus); the incidence of the cleft phallus in this group was 25% (3 cases in one litter).

Table 1. Malformation and Inflammatory Lesion in Genital Organ of Male Rats Prenatally Exposed to Chemicals

Chemical	Dosing period	Group	Males	Litters	Cleft prepuce	Cleft phallus	Ectopic testis	Hypoplasia of prostate	Prostatitis/ Vesiculitis
FLU	GD 14-17	Control	12	3	0	0	0	0	0
		1 mg/kg	12	3	0	0	0	0	0
		10 mg/kg	12	3	3 (2)	7 (3)	2 (2)	0	0
	GD 18-21	Control	12	3	0	0	0	0	0
		1 mg/kg	12	3	0	0	0	0	0
		10 mg/kg	12	3	0	3 (1)	0	0	7 (3)
DDE	GD 14-17	Control	12	3	0	0	0	0	0
		10 mg/kg	12	3	0	0	0	0	0
		30 mg/kg	16	4	0	0	0	0	0
		100 mg/kg	16	4	0	0	0	0	0
	GD 18-21	Control	14	4	0	0	0	0	0
		10 mg/kg	16	4	0	0	0	0	0
		30 mg/kg	14	4	0	0	0	0	0
		100 mg/kg	4	1	0	0	0	0	0
VZ	GD 14-17	Control	20	5	0	0	0	0	0
		10 mg/kg	20	5	0	0	0	0	0
		30 mg/kg	20	5	0	0	0	0	0
		100 mg/kg	20	5	17 (5)	17 (5)	1 (1)	1 (1)	0
	GD 18-21	Control	20	5	0	0	0	0	0
		10 mg/kg	16	4	0	0	1 (1)	0	0
		30 mg/kg	16	4	0	0	0	0	0
		100 mg/kg	15	4	0	0	0	0	0
DES	GD 14-17	Control	19	5	0	0	0	0	0
		0.1 μg/kg	12	3	0	0	0	0	0
		1 μg/kg	20	5	0	0	0	0	0
		10 μg/kg	16	4	0	0	0	0	0
		100 μg/kg	16	4	0	0	0	0	0
		300 μg/kg	12	4	0	0	0	0	0
	GD 18-21	Control	16	4	0	0	0	0	0
		0.1 μg/kg	20	5	0	0	0	0	0
		1 μg/kg	16	4	0	0	0	0	0
		10 μg/kg	20	5	0	0	0	0	0
		100 μg/kg	20	5	0	0	0	0	0
		300 μg/kg	7	2	0	0	0	1 (1)	0
EE	GD 14-17	Control	16	4	0	0	0	0	0
		10 μg/kg	16	4	0	0	0	0	0
		100 μg/kg	20	5	0	0	0	0	0
	GD 18-21	Control	19	5	0	0	0	0	0
		10 μg/kg	15	4	0	0	0	0	0
		100 μg/kg	16	4	0	0	0	0	0
TAM	GD 14-17	Control	16	4	0	0	0	0	0
		0.03 mg/kg	15	4	0	0	0	0	0
		0.1 mg/kg	7	2	0	0	0	0	0
	GD 18-21	Control	19	5	0	0	0	0	0
		0.03 mg/kg	12	3	0	0	0	0	0
		0.1 mg/kg	11	3	0	0	0	0	0

Value: number of cases (litters) with abnormality.

FLU: flutamide; DDE: *p,p'*-dichlorodiphenyldichloroethylene; VZ: vinclozolin; DES: diethylstilbestrol; EE: ethynylestradiol; TAM: tamoxifen; GD: gestational day.

Cleft prepuce and cleft phallus were also observed in males from dams exposed to 100 mg/kg of VZ on GD 14–17 (Figs. 2C, 2D). The incidence of cleft prepuce and cleft phallus was 85% (17 cases in all of the 5 litters). However, there was no cleft at the prepuce or phallus of males from dams exposed to VZ on GD 18–21 (Figs. 2E, 2F). The time of sexual maturation is determined by complete separation of the prepuce from the ventral surface of the glans penis, but preputial separation could not determined in males with cleft phallus, since complete separation in the glans penis was not evident.

The testis of males from dams exposed to 10 mg/kg of

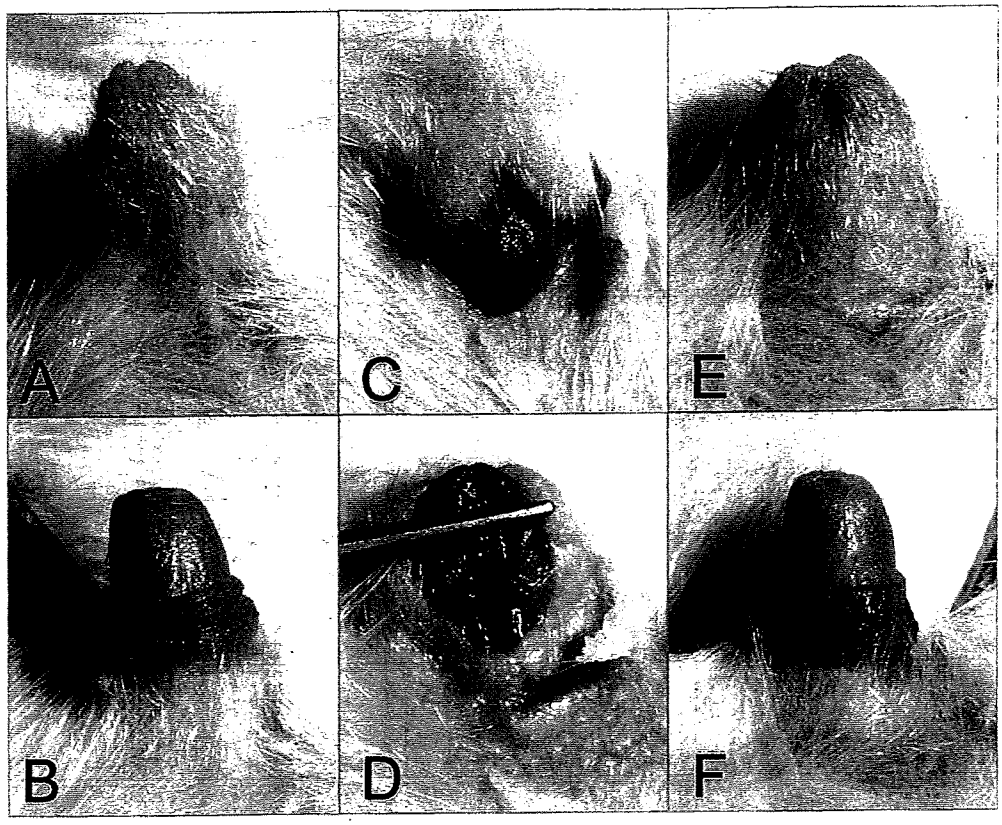

Fig. 2. External genitalia of male rats on PND 56.
A and B: Control male rat. Prepuce is completely retracted. C and D: Male rat from a dam exposed to 100 mg/kg vinclozolin on GD 14–17. Ventral side of the prepuce has a cleft and the glans penis is observed from the cleft (C). The ventral part of the penis is incompletely formed (cleft phallus, shown in D). E and F: Male rat from a dam exposed to 100 mg/kg vinclozolin on GD 18–21. There was no cleft on the ventral surface of the prepuce or penis.

FLU on GD 14–17 (17%, 2 cases in 2 litters) or 100 mg/kg of VZ on GD 14–17 (5%, one case in one litter) did not descend into the scrotum and was located in the ventral subcutis (ectopic testis) instead. One male from a dam exposed to 10 mg/kg VZ on GD 18–21 also had an ectopic testis. Ectopic testis was observed unilaterally. Marked inflammation in the prostate and seminal vesicle occurred in the group exposed to 10 mg/kg FLU on GD 18–21, and 5 of 12 males in this group died from the severe inflammation. Males with marked prostatitis and vesiculitis did not show hypospadias in their glans penis.

3) Preputial separation

Males with hypospadias were excluded from the preputial separation analysis, and the resulting days of preputial separation of males without hypospadias are summarized in Table 2. Preputial separation in males from dams exposed to 10 mg/kg of FLU was significantly delayed in both GD 14–17 and GD 18–21 treatment groups, and 2 males in the GD 18–21 treatment group had incomplete preputial separation on the day of autopsy, PND 56. In the group exposed to 100 mg/kg of DDE on GD 18–21, all pups of 3 litters died. There were no differences among the other 3 groups. In the 100 mg/kg VZ groups, male pups without hypospadias showed no difference from the control group, and the lower dose groups did not show any significant difference from the control. Two of 4 pregnant females administered 300 μg/kg of DES from GD 18 were excluded from the experiment because these females delivered their pups on GD 21 before the end of the administration period. Preputial separation of male offspring exposed to DES was not different from the control group. EE and TAM administered in any exposure period did not affect preputial separation.

Body weight on the day of complete preputial separation was significantly higher in males from dams exposed to FLU on GD 18–21, but there were no changes in other chemically-treated groups.

4) Measurement of AGD on PND 6

Table 3 shows the AGD, body weight, and correction values of AGD [AGD (mm)/ $\sqrt[3]{\text{body weight (g)}}$ of pups] on PND 6. Correction values of AGD were significantly decreased in the groups exposed to 10 mg/kg FLU on GD 14–17 or GD 18–21, and the group exposed to 100 mg/kg VZ on GD 14–17. DES induced a significant reduction in AGD of the 100 and 300 μg/kg groups exposed on GD 18–21. DDE, EE and TAM did not induce a significant

Table 2. Preputial Separation and Body Weights of Male Rats Prenatally Exposed to Chemicals

Chemical	Dosing period	Group	Males	Litters	PND of preputial separation	BW (g) on the day of PPS	Incomplete separation
FLU	GD 14-17	Control	12	3	42.8 ± 2.2	232.2 ± 26.5	0
		1 mg/kg	12	3	42.4 ± 0.3	216.5 ± 3.3	0
		10 mg/kg	5	2	49.1 ± 0.6 **	271.2 ± 41.3	0
	GD 18-21	Control	12	3	43.2 ± 1.4	227.0 ± 19.2	0
		1 mg/kg	12	3	44.5 ± 0.7	249.8 ± 12.7	0
		10 mg/kg	5	2	51.3 ± 0.5 **	322.2 ± 13.7 **	2 (1)
DDE	GD 14-17	Control	12	3	42.8 ± 2.2	232.2 ± 26.5	0
		10 mg/kg	12	3	42.6 ± 0.4	242.1 ± 23.3	0
		30 mg/kg	16	4	43.1 ± 1.0	248.3 ± 20.1	0
		100 mg/kg	16	4	44.6 ± 3.0	242.5 ± 14.0	0
	GD 18-21	Control	14	4	43.8 ± 1.5	231.7 ± 26.7	0
		10 mg/kg	16	4	43.3 ± 1.6	233.7 ± 19.6	0
		30 mg/kg	14	4	43.0 ± 1.0	239.3 ± 16.2	0
		100 mg/kg	4	1	43.5	233.5	0
VZ	GD 14-17	Control	20	5	44.4 ± 1.2	245.3 ± 10.5	0
		10 mg/kg	20	5	43.7 ± 1.6	243.7 ± 16.4	0
		30 mg/kg	20	5	44.5 ± 1.7	246.2 ± 13.7	0
		100 mg/kg	3	1	43.7	254.1	0
	GD 18-21	Control	20	5	44.1 ± 0.3	252.1 ± 36.7	0
		10 mg/kg	16	4	43.8 ± 1.5	248.2 ± 17.3	0
		30 mg/kg	16	4	43.3 ± 1.9	240.8 ± 11.4	0
		100 mg/kg	15	4	45.1 ± 1.7	259.2 ± 22.3	0
DES	GD 14-17	Control	19	5	44.4 ± 1.7	246.4 ± 12.9	0
		0.1 μg/kg	12	3	43.3 ± 0.5	242.3 ± 12.7	0
		1 μg/kg	20	5	43.3 ± 1.3	243.3 ± 17.6	0
		10 μg/kg	16	4	42.8 ± 1.3	244.2 ± 19.9	0
		100 μg/kg	16	4	43.7 ± 1.4	257.9 ± 8.4	0
		300 μg/kg	12	4	46.1 ± 1.0	260.8 ± 20.5	0
	GD 18-21	Control	16	4	44.6 ± 1.1	246.5 ± 16.0	0
		0.1 μg/kg	20	5	45.1 ± 1.1	255.9 ± 22.9	0
		1 μg/kg	16	4	43.3 ± 1.2	252.5 ± 18.5	0
		10 μg/kg	20	5	43.4 ± 1.4	241.6 ± 7.5	0
		100 μg/kg	20	5	44.0 ± 0.6	245.3 ± 9.1	0
		300 μg/kg	7	2	46.8 ± 1.1	239.9 ± 1.7	0
EE	GD 14-17	Control	16	4	44.4 ± 1.0	254.6 ± 11.9	0
		10 μg/kg	16	4	45.6 ± 2.0	240.2 ± 6.2	0
		100 μg/kg	20	5	45.8 ± 2.2	261.6 ± 20.2	0
	GD 18-21	Control	19	5	43.6 ± 1.7	246.6 ± 9.6	0
		10 μg/kg	15	4	43.3 ± 0.5	248.3 ± 10.1	0
		100 μg/kg	16	4	42.8 ± 1.6	247.6 ± 12.3	0
TAM	GD 14-17	Control	16	4	44.4 ± 1.0	254.6 ± 11.9	0
		0.03 mg/kg	15	4	44.5 ± 1.3	252.7 ± 25.2	0
		0.1 mg/kg	7	2	43.5 ± 0.3	244.2 ± 11.6	0
	GD 18-21	Control	19	5	43.6 ± 1.7	246.6 ± 9.6	0
		0.03 mg/kg	12	3	43.2 ± 1.0	242.7 ± 32.3	0
		0.1 mg/kg	11	3	43.7 ± 0.6	257.5 ± 30.3	0

Value: Mean ± S.D. calculated using the litter as the unit.

FLU: flutamide; DDE: *p,p'*-dichlorodiphenyldichloroethylene; VZ: vinclozolin, DES: diethylstilbestrol; EE: ethynylestradiol; TAM: tamoxifen; GD: gestational day; PND: postnatal day; BW: body weight; PPS: preputial separation.

Incomplete separation: number of animals (litters) with incomplete separation on PND 56.

**: significantly different from control, $p<0.01$.

Table 3. Anogenital Distance (AGD) of PND 6 Male Rats Prenatally Exposed to Chemicals

Chemical	Dosing period	Group	Males	Litters	AGD (mm)	Correction value of AGD (mm/$^3\sqrt{g}$)	Body weight (g)
FLU	GD 14-17	Control	12	3	7.07 ± 0.59	2.85 ± 0.11	15.4 ± 3.1
		1 mg/kg	20	3	6.21 ± 0.09	2.63 ± 0.03	13.3 ± 1.0
		10 mg/kg	26	3	5.53 ± 0.39 **	2.35 ± 0.12 **	13.0 ± 1.0
	GD 18-21	Control	19	3	6.70 ± 0.25	2.93 ± 0.07	11.9 ± 0.7
		1 mg/kg	20	3	7.04 ± 0.71	2.89 ± 0.21	14.4 ± 1.3
		10 mg/kg	22	3	5.82 ± 0.45	2.42 ± 0.22 *	14.1 ± 1.2
DDE	GD 14-17	Control	12	3	7.07 ± 0.59	2.85 ± 0.11	15.4 ± 3.1
		10 mg/kg	23	3	6.84 ± 0.38	2.84 ± 0.01	14.0 ± 2.2
		30 mg/kg	30	4	6.67 ± 0.59	2.78 ± 0.13	13.8 ± 2.3
		100 mg/kg	23	4	6.32 ± 0.44	2.71 ± 0.11	12.8 ± 1.6
	GD 18-21	Control	19	4	7.55 ± 0.86	3.10 ± 0.20	14.5 ± 2.3
		10 mg/kg	26	4	6.96 ± 0.56	2.93 ± 0.20	13.4 ± 1.1
		30 mg/kg	26	4	7.29 ± 0.45	3.00 ± 0.09	14.5 ± 2.9
		100 mg/kg	5	1	7.40	3.04	14.5
VZ	GD 14-17	Control	35	5	6.75 ± 0.39	2.86 ± 0.14	13.2 ± 0.9
		10 mg/kg	38	5	6.60 ± 0.14	2.72 ± 0.08	14.5 ± 1.9
		30 mg/kg	28	5	6.46 ± 0.22	2.73 ± 0.08	13.2 ± 0.4
		100 mg/kg	34	5	4.87 ± 0.48 **	2.02 ± 0.22 **	14.1 ± 2.2
	GD 18-21	Control	43	5	6.25 ± 0.75	2.65 ± 0.29	13.2 ± 2.0
		10 mg/kg	28	4	6.14 ± 0.38	2.59 ± 0.15	13.3 ± 0.8
		30 mg/kg	24	4	6.59 ± 0.31	2.70 ± 0.06	14.7 ± 1.8
		100 mg/kg	26	4	5.74 ± 0.54	2.41 ± 0.20	13.6 ± 1.4
DES	GD 14-17	Control	36	5	6.25 ± 0.41	2.66 ± 0.06	13.0 ± 1.9
		0.1 μg/kg	20	3	6.35 ± 0.13	2.60 ± 0.09	14.6 ± 0.8
		1 μg/kg	45	5	6.35 ± 0.20	2.66 ± 0.06	13.8 ± 1.7
		10 μg/kg	21	4	6.64 ± 0.54	2.75 ± 0.14	14.1 ± 1.8
		100 μg/kg	26	4	6.40 ± 0.62	2.63 ± 0.19	14.4 ± 1.3
		300 μg/kg	17	4	5.77 ± 0.83	2.54 ± 0.30	11.6 ± 0.9
	GD 18-21	Control	26	4	6.35 ± 0.46	2.68 ± 0.15	13.3 ± 0.9
		0.1 μg/kg	44	5	6.22 ± 0.26	2.64 ± 0.09	13.2 ± 1.4
		1 μg/kg	35	4	6.28 ± 0.28	2.63 ± 0.06	13.7 ± 2.2
		10 μg/kg	36	5	6.17 ± 0.31	2.62 ± 0.09	13.1 ± 0.9
		100 μg/kg	27	5	5.47 ± 0.44 **	2.38 ± 0.10 **	12.2 ± 1.6
		300 μg/kg	9	2	4.45 ± 0.35 **	2.08 ± 0.16 **	9.7 ± 0.0
EE	GD 14-17	Control	30	4	6.73 ± 0.60	2.82 ± 0.13	13.6 ± 2.1
		10 μg/kg	26	4	6.16 ± 0.52	2.76 ± 0.15	11.1 ± 1.2
		100 μg/kg	37	5	6.58 ± 0.19	2.80 ± 0.07	13.0 ± 1.0
	GD 18-21	Control	32	5	6.78 ± 0.41	2.81 ± 0.15	14.1 ± 0.6
		10 μg/kg	35	4	6.98 ± 0.34	3.00 ± 0.16	12.6 ± 0.5
		100 μg/kg	31	5	7.07 ± 0.54	2.89 ± 0.22	14.7 ± 1.5
TAM	GD 14-17	Control	30	4	6.73 ± 0.60	2.82 ± 0.13	13.6 ± 2.1
		0.03 mg/kg	25	4	6.50 ± 0.88	2.71 ± 0.21	13.8 ± 2.7
		0.1 mg/kg	9	2	6.52 ± 0.19	2.73 ± 0.02	13.7 ± 0.9
	GD 18-21	Control	32	5	6.78 ± 0.41	2.81 ± 0.15	14.1 ± 0.6
		0.03 mg/kg	16	3	6.84 ± 0.06	2.73 ± 0.12	15.8 ± 1.7
		0.1 mg/kg	25	4	6.57 ± 0.46	2.68 ± 0.14	15.0 ± 2.6

Value: Mean ± S.D. calculated using the litter as the unit.

FLU: flutamide; DDE: *p,p'*-dichlorodiphenyldichloroethylene; VZ: vinclozolin; DES: diethylstilbestrol; EE: ethynylestradiol; TAM: tamoxifen; GD: gestational day; PND: postnatal day.

Correction value of AGD: AGD(mm)/ $\sqrt[3]{\text{body weight (g)}}$.

*: significantly different from control, $p<0.05$; **: significantly different from control, $p<0.01$.

Table 4. Relative Organ Weights and Body Weights of PND 56 Male Rats Prenatally Exposed to Chemicals

Chemical Dosing period	Group	Males	Litters	Body weight (g)	Testes (mg/g)	Epididymides (mg/g)	Ventral prostate (mg/g)	Seminal Vesicles (mg/g)
FLU								
GD 14-17	Control	12	3	350.2 ± 10.6	8.674 ± 0.105	1.439 ± 0.054	0.791 ± 0.007	1.464 ± 0.188
	1 mg/kg	12	3	331.1 ± 2.2	8.433 ± 0.313	1.481 ± 0.026	0.829 ± 0.060	1.605 ± 0.065
	10 mg/kg	12	3	341.4 ± 27.5	7.982 ± 0.819	1.410 ± 0.091	0.818 ± 0.105	1.657 ± 0.284
GD 18-21	Control	12	3	333.0 ± 15.5	8.364 ± 0.084	1.499 ± 0.066	0.855 ± 0.023	1.651 ± 0.227
	1 mg/kg	12	3	350.0 ± 21.9	8.164 ± 0.115	1.456 ± 0.083	0.798 ± 0.143	1.694 ± 0.164
	10 mg/kg	7	3	346.6 ± 29.3	8.447 ± 0.438	1.460 ± 0.080	0.641 ± 0.115	1.642 ± 0.278
DDE								
GD 14-17	Control	12	3	350.2 ± 10.6	8.674 ± 0.105	1.439 ± 0.054	0.791 ± 0.007	1.464 ± 0.188
	10 mg/kg	12	3	359.1 ± 38.6	8.330 ± 0.125	1.447 ± 0.113	0.862 ± 0.092	1.703 ± 0.206
	30 mg/kg	16	4	368.9 ± 38.3	7.763 ± 0.492**	1.431 ± 0.062	0.946 ± 0.082	1.672 ± 0.201
	100 mg/kg	16	4	340.4 ± 23.4	8.785 ± 0.229	1.465 ± 0.097	0.743 ± 0.102	1.601 ± 0.280
GD 18-21	Control	14	4	338.5 ± 15.6	8.291 ± 0.374	1.444 ± 0.095	0.882 ± 0.062	1.817 ± 0.032
	10 mg/kg	16	4	342.9 ± 15.6	8.308 ± 0.186	1.490 ± 0.058	0.758 ± 0.052	1.794 ± 0.270
	30 mg/kg	14	4	352.7 ± 21.6	7.927 ± 0.285	1.443 ± 0.075	0.903 ± 0.120	1.785 ± 0.142
	100 mg/kg	4	1	339.9	9.027	1.582	1.003	1.816
VZ								
GD 14-17	Control	20	5	355.2 ± 19.5	7.177 ± 0.212	1.463 ± 0.073	0.982 ± 0.089	1.729 ± 0.262
	10 mg/kg	19	5	352.9 ± 6.1	7.486 ± 0.358	1.514 ± 0.080	0.931 ± 0.047	1.752 ± 0.068
	30 mg/kg	19	5	344.6 ± 18.9	7.809 ± 0.699	1.489 ± 0.139	0.992 ± 0.124	1.840 ± 0.308
	100 mg/kg	20	1	369.2 ± 6.4	7.404 ± 0.608	1.489 ± 0.062	0.755 ± 0.093**	1.675 ± 0.213
GD 18-21	Control	20	5	363.8 ± 49.2	7.766 ± 0.955	1.523 ± 0.169	0.970 ± 0.131	1.869 ± 0.210
	10 mg/kg	16	4	358.5 ± 32.8	7.768 ± 0.493	1.596 ± 0.166	1.007 ± 0.178	1.996 ± 0.327
	30 mg/kg	16	4	357.4 ± 16.1	7.525 ± 0.405	1.597 ± 0.084	0.934 ± 0.095	1.830 ± 0.339
	100 mg/kg	15	4	357.2 ± 19.7	7.609 ± 0.309	1.517 ± 0.066	0.963 ± 0.118	1.771 ± 0.119
DES								
GD 14-17	Control	19	5	347.0 ± 23.9	7.980 ± 1.152	1.454 ± 0.106	0.983 ± 0.132	1.922 ± 0.086
	0.1 μg/kg	12	3	353.6 ± 11.7	7.802 ± 0.485	1.581 ± 0.043	1.091 ± 0.147	1.902 ± 0.239
	1 μg/kg	20	5	358.3 ± 22.7	7.639 ± 0.619	1.527 ± 0.105	1.004 ± 0.081	1.850 ± 0.092
	10 μg/kg	16	4	363.0 ± 23.6	7.966 ± 0.554	1.626 ± 0.088	1.037 ± 0.102	2.120 ± 0.187
	100 μg/kg	16	4	373.5 ± 13.7	7.337 ± 0.523	1.532 ± 0.084	0.913 ± 0.029	1.888 ± 0.243
	300 μg/kg	12	4	350.4 ± 20.1	7.888 ± 0.320	1.592 ± 0.062	0.718 ± 0.058**	1.885 ± 0.251
GD 18-21	Control	16	4	343.8 ± 11.2	8.006 ± 0.610	1.609 ± 0.102	1.115 ± 0.040	1.872 ± 0.144
	0.1 μg/kg	20	5	353.3 ± 20.7	8.369 ± 1.418	1.491 ± 0.072	1.100 ± 0.199	1.853 ± 0.263
	1 μg/kg	16	4	370.1 ± 25.7	7.110 ± 0.642	1.517 ± 0.161	1.033 ± 0.094	1.866 ± 0.238
	10 μg/kg	20	5	354.8 ± 15.0	7.959 ± 0.389	1.633 ± 0.116	1.050 ± 0.113	1.974 ± 0.290
	100 μg/kg	20	5	354.9 ± 10.2	7.457 ± 0.337	1.577 ± 0.079	0.753 ± 0.081**	1.743 ± 0.136
	300 μg/kg	7	2	316.5 ± 2.8	8.228 ± 0.183	1.664 ± 0.025	0.668 ± 0.134**	1.991 ± 0.364
EE								
GD 14-17	Control	16	4	364.1 ± 19.7	7.912 ± 0.032	1.583 ± 0.122	0.954 ± 0.095	1.918 ± 0.116
	10 μg/kg	16	4	333.8 ± 21.5	7.693 ± 0.280	1.477 ± 0.076	0.967 ± 0.145	1.877 ± 0.185
	100 μg/kg	20	5	354.9 ± 17.0	7.745 ± 0.189	1.538 ± 0.086	0.980 ± 0.165	1.993 ± 0.197
GD 18-21	Control	19	5	358.5 ± 14.1	7.677 ± 0.424	1.477 ± 0.112	0.949 ± 0.092	1.967 ± 0.185
	10 μg/kg	15	4	372.0 ± 16.2	7.752 ± 0.454	1.539 ± 0.073	1.036 ± 0.124	2.106 ± 0.062
	100 μg/kg	16	4	364.8 ± 6.2	7.791 ± 0.475	1.618 ± 0.062	0.923 ± 0.089	2.070 ± 0.173
TAM								
GD 14-17	Control	16	4	364.1 ± 19.7	7.912 ± 0.032	1.583 ± 0.122	0.954 ± 0.095	1.918 ± 0.116
	0.03 mg/kg	15	4	356.8 ± 26.7	7.808 ± 0.311	1.491 ± 0.116	0.996 ± 0.159	1.948 ± 0.285
	0.1 mg/kg	7	2	360.7 ± 13.4	7.320 ± 0.038	1.458 ± 0.117	0.903 ± 0.014	1.659 ± 0.076
GD 18-21	Control	19	5	358.5 ± 14.1	7.677 ± 0.424	1.477 ± 0.112	0.949 ± 0.092	1.967 ± 0.185
	0.03 mg/kg	12	3	359.8 ± 44.0	7.142 ± 0.660	1.469 ± 0.030	0.932 ± 0.174	1.869 ± 0.277
	0.1 mg/kg	11	3	376.4 ± 49.8	7.475 ± 0.202	1.513 ± 0.020	0.949 ± 0.036	1.731 ± 0.093

Value: Mean ± S.D. calculated using the litter as the unit.

FLU: flutamide; DDE: *p,p'*-dichlorodiphenyldichloroethylene; VZ: vinclozolin; DES: diethylstilbestrol; EE: ethynylestradiol; TAM: tamoxifen; GD: gestational day; PND: postnatal day.

**: significantly different from control, $p<0.01$.

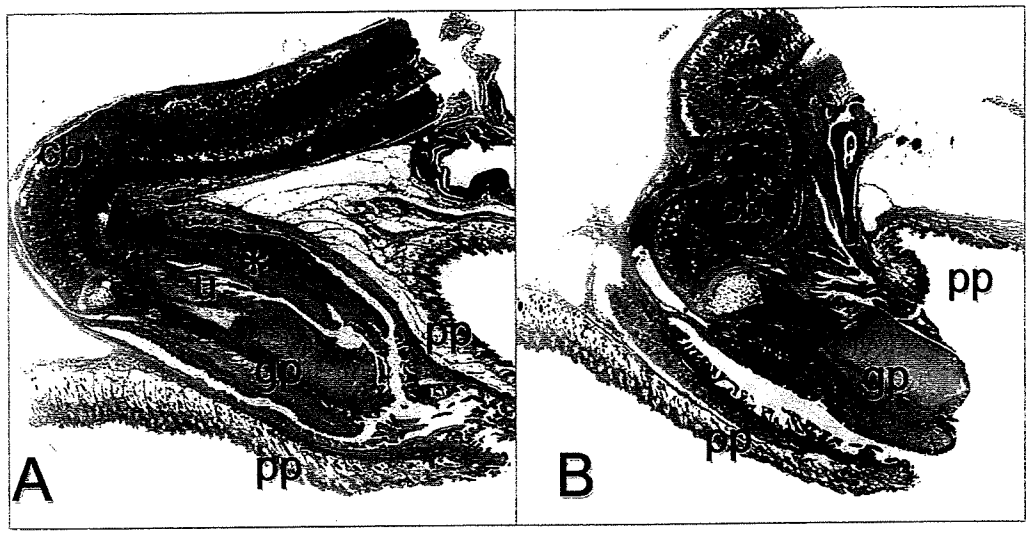

Fig. 3. Sagittal sections of the prepuce and penis of male rats sacrificed on PND 56.
A: Control male rat. The prepuce (pp) is separated from the glans penis (gp). The urethra (u) is located in the center of the glans penis. *: ventral half of the glans penis. cb: cavernous body. H&E staining, magnification: × 6. B: Male rat from a dam exposed to 100 mg/kg vinclozolin on GD 14–17. The prepuce (pp) is separated from the glans penis (gp) at the dorsal part, and the urethra (u) is located between the glans penis and subcutis. The prepuce is hypoplastic and the glans penis is not completely covered by the prepuce. The cavernous body (cb) of the penis is tortuous and bent. H&E staining, magnification: × 6.

difference in AGD. Body weights at the time of AGD measurement did not show any significant difference.

5) Organ weight

Relative organ weights of males prenatally exposed to chemicals are shown in Table 4. Changes in absolute organ weights were similar to changes in relative organ weights. Relative weights of the ventral prostate were decreased significantly in males from dams exposed to 100 mg/kg of VZ on GD 14–17, 300 μg/kg of DES on GD 14–17, and 100 and 300 μg/kg of DES on GD 18–21. Although there was a significant decrease in relative organ weight of the testes in the DDE group, a dose dependent relationship was not detected.

6) Pathological examination

Histopathological examination of representative males from dams exposed to 10 mg/kg of FLU or 100 mg/kg of VZ on GD 14–17 revealed a defect in the ventral half of the glans penis (cleft phallus). The urethra was not located in the center of the glans penis, but instead was observed at the ventral surface of the penis (Fig. 3B). The dorsal surface of the glans penis and prepuce of PND 56 males were covered with keratinized stratified squamous epithelium, and the prepuce was separated from the glans penis. The ventral part of the glans penis and ventral epithelium were not formed between the urethra and subcutis, the ventral surface of the glans penis was not covered with squamous epithelium, and preputial separation did not progress at the ventral aspect. The external urethral orifice opened at the ventral surface of the penis (hypospadias). These males with cleft phallus showed a tortuous cavernous body of the penis (Fig. 3B). On PND 6, hypoplasia of the ventral half of the glans penis and tortuous cavernous body were observed in male pups from dams exposed to 100 mg/kg of VZ on GD 14–17 (Fig. 4B).

Ectopic testes were induced by FLU and VZ, and showed severe atrophy of the seminiferous tubule in PND 56 males. Severe prostatitis and seminal vesiculitis were observed in males from dams exposed to 10 mg/kg of FLU on GD 18–21. Neutrophils, lymphocytes and macrophages infiltrated the prostate, seminal vesicles, and surrounding tissues of five animals which died (PND 44, 45, 52, 55 and 56) and two sacrificed (PND 56) males. In some cases, hemorrhage was observed in the muscular layer of the urinary bladder. The males with severe prostatitis and vesiculitis did not show hypoplasia in the ventral half of the glans penis.

Postnatal exposure

1) Preputial separation

The day of preputial separation and the body weights on the day of preputial separation were statistically analyzed using both the litter as the unit and the individual data. There were no significant changes on the day of preputial separation of males exposed to FLU, DDE or VZ on PND 1–5 (Table 5). On the other hand, the day of preputial separation was significantly delayed in groups exposed to 10 and 30 mg/kg of FLU and 30 mg/kg of VZ on PND 35–39. Statistical analyses using the individual data revealed additional significances in the delay of preputial separation in the groups exposed 300 mg/kg of DDE on PND 17–21 and 100 mg/kg on PND 35–39, and 100 mg/kg of VZ on PND 35–39. In the DDE group, 10 of 12 males exposed to 300 mg/kg of DDE on PND 35–39 died before maturation.

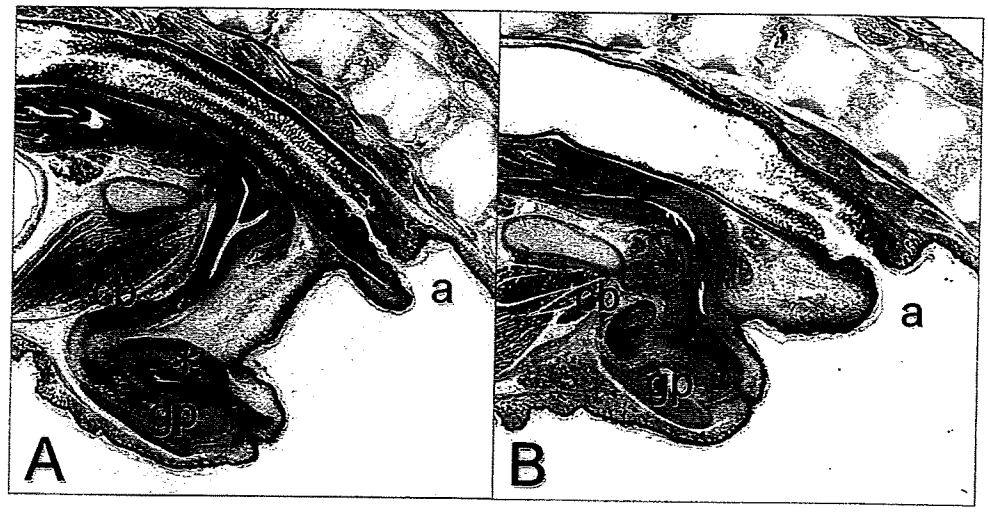

Fig. 4. Sagittal sections of the genital tubercle of males sacrificed on PND 6.
A: Control male rat. Glans penis (gp) is bordered with dorsal and ventral epithelium. The urethra is located in the center of the glans penis. a: anus. *: ventral half of the glans penis. H&E staining, magnification: × 8. B: A male rat from a dam exposed to 100 mg/kg vinclozolin on GD 14–17. The dorsal part of the glans penis is bordered with epithelium. The urethra is observed at the ventral surface of the glans penis (gp) and the cavernous body (cb) shows tortuous structure. a: anus. H&E staining, magnification: × 8.

Preputial separation in two surviving males (PND 48 and 50) was delayed compared to controls. A significant delay was not detected in males exposed to FLU or VZ on PND 17–21.

DES induced a significant delay in the 300 μg/kg group exposed on PND 1–5 or PND 35–39, while 2 of 16 males in the 100 μg/kg group and 3 of 16 males in the 300 μg/kg group exposed on PND 1–5 showed incomplete preputial separation on PND 56. In statistical analyses using the individual data, significant delays of preputial separation were also observed in the group exposed to 100 μg/kg of DES on PND 1–5 or 35–39. There were no significant delays in males exposed on PND 17–21.

In EE treatment, preputial separation was significantly delayed in males of the 100 μg/kg group exposed on PND 1–5, and 1 of 12 males in the 10 μg/kg group and 10 of 12 males in the 100 μg/kg group had incomplete separation on PND 56. Administration of 10 μg/kg of EE on PND 6–10 or PND 11–15 was added to the experimental protocol to define the sensitive period, because PND 1–5 exposure induced a marked effect on preputial separation. Although a delay in preputial separation was observed after PND 6–10 exposure, there was no significant difference between the PND 11–15 exposed group and controls. The 3 mg/kg TAM treated groups exposed on PND 1–5 also showed a significant delay in preputial separation, and 5 of 16 males in the 1 mg/kg group and 14 of 15 males in the 3 mg/kg group had incomplete separation on PND 56. Slight delay in preputial separation was also observed in the 0.3 mg/kg TAM group, and 2 of 16 males had incomplete separation on PND 56. In the statistical analyses using the individual data, significant delays of preputial separation were also observed in the groups exposed to 10 μg/kg of EE and 1 mg/kg of TAM on PND 1–5. No influence of EE and TAM treatments on PND 17–21 or PND 35–39 was detected.

Body weights on the day of preputial separation showed a higher value in the groups with a delay of separation.

2) Organ weight

Relative organ weights of males postnatally exposed to the chemicals are presented in Table 6 using the litter as the unit and in Table 7 using the individual data. Absolute organ weights showed similar changes to those of relative organ weights. Relative weights of the ventral prostate were decreased significantly in males exposed to 30 mg/kg of FLU on PND 35–39. No significant changes were observed in males exposed to FLU on PND 1–5 or 17–21, and DDE and VZ in any period.

DES caused a significant reduction in the relative weight of the ventral prostate in the 100 and 300 μg/kg groups exposed on PND 1–5. No significant changes were observed in males exposed to DES on PND 17–21 or PND 35–39. EE caused reductions in the testes, ventral prostate and seminal vesicles of males in the 100 μg/kg group and seminal vesicles of males in the 10 μg/kg group exposed to EE on PND 1–5. No significant changes in relative weight were observed in males exposed on PND 17–21 or PND 35–39. TAM treatment on PND 1–5 led to reductions in body and reproductive organ weights in the 3 mg/kg group and the ventral prostate weight in the 1 mg/kg group. No weight reductions were observed in the reproductive organs of males exposed to TAM on PND 17–21 or PND 35–39. Although the statistical analyses using the individual data showed significant results in the lower dose group and the other organs, there were no significances in the groups exposed to VZ.

Table 5. Preputial Separation and Body Weights of Male Rats Postnatally Exposed to Chemicals

Chemical	Dosing period	Group	Litters	PND of preputial separation	BW (g) on the day of PPS	Males	PND of preputial separation	BW (g) on the day of PPS	Incomplete separation
FLU	PND 1-5	Control	4	44.4 ± 2.3	268.4 ± 8.9	16	44.4 ± 2.4	268.4 ± 21.2	0
		1 mg/kg	4	42.7 ± 2.4	246.5 ± 21.6	16	42.7 ± 2.3	246.5 ± 22.4	0
		10 mg/kg	4	42.8 ± 2.1	257.6 ± 15.2	16	42.8 ± 2.1	257.6 ± 21.4	0
		30 mg/kg	4	43.9 ± 1.1	252.7 ± 19.6	15	44.0 ± 2.0	252.7 ± 27.9	0
	PND 17-21	Control	3	44.3 ± 0.4	258.3 ± 7.9	12	44.3 ± 1.4	258.3 ± 19.0	0
		1 mg/kg	3	45.0 ± 2.9	256.7 ± 20.1	12	45.0 ± 3.2	256.7 ± 29.7	0
		10 mg/kg	3	43.2 ± 1.9	250.6 ± 17.5	12	43.2 ± 2.0	250.6 ± 23.5	0
		30 mg/kg	3	43.6 ± 2.5	256.5 ± 28.2	12	43.6 ± 2.5	256.5 ± 26.9	0
	PND 35-39	Control	4	43.5 ± 1.4	253.1 ± 16.9	16	43.5 ± 1.7	253.1 ± 18.6	0
		1 mg/kg	4	44.6 ± 0.8	272.2 ± 9.6	16	44.6 ± 1.4	272.2 ± 20.4	0
		10 mg/kg	4	46.8 ± 1.2**	287.8 ± 9.2*	15	46.8 ± 2.0**	287.9 ± 19.6**	0
		30 mg/kg	4	47.1 ± 1.4**	294.5 ± 24.7*	16	47.1 ± 2.2**	294.5 ± 30.0**	0
DDE	PND 1-5	Control	3	42.3 ± 1.1	239.8 ± 8.6	12	42.3 ± 1.7	239.8 ± 13.5	0
		10 mg/kg	3	42.0 ± 0.7	243.3 ± 8.4	12	42.0 ± 1.3	243.3 ± 17.5	0
		30 mg/kg	3	40.9 ± 1.2	227.3 ± 7.5	12	40.9 ± 1.7	227.3 ± 15.4	0
		100 mg/kg	3	42.1 ± 0.2	242.2 ± 14.3	11	42.1 ± 1.2	240.8 ± 19.9	0
		300 mg/kg	3	42.2 ± 0.6	248.5 ± 13.5	12	42.2 ± 0.9	248.5 ± 17.8	0
	PND 17-21	Control	3	43.6 ± 0.1	243.4 ± 2.6	12	43.6 ± 0.8	243.4 ± 16.2	0
		10 mg/kg	3	43.8 ± 0.9	256.4 ± 10.5	12	43.8 ± 1.4	256.4 ± 15.5	0
		30 mg/kg	3	43.9 ± 1.1	246.1 ± 16.9	11	43.9 ± 1.6	245.1 ± 21.2	0
		100 mg/kg	3	44.5 ± 0.3	246.7 ± 3.8	12	44.5 ± 1.3	246.7 ± 19.5	0
		300 mg/kg	4	45.6 ± 1.4	261.0 ± 20.3	13	45.5 ± 1.4**	259.1 ± 21.5	0
	PND 35-39	Control	3	42.1 ± 1.2	210.0 ± 21.7	12	42.1 ± 1.3	210.0 ± 22.2	0
		10 mg/kg	3	43.1 ± 1.4	238.4 ± 19.7	12	43.1 ± 1.3	238.4 ± 20.0**	0
		30 mg/kg	3	43.4 ± 2.7	232.3 ± 11.1	12	43.4 ± 2.5	232.3 ± 18.1*	0
		100 mg/kg	3	46.4 ± 3.1	264.7 ± 5.1**	12	46.4 ± 3.3**	264.7 ± 16.4**	0
VZ	PND 1-5	Control	4	41.9 ± 0.9	244.3 ± 9.5	16	41.9 ± 1.5	244.3 ± 16.3	0
		10 mg/kg	3	42.8 ± 0.9	253.7 ± 19.0	12	42.8 ± 1.4	253.7 ± 24.9	0
		30 mg/kg	4	42.5 ± 1.6	252.7 ± 17.9	15	42.3 ± 1.7	251.2 ± 19.2	0
		100 mg/kg	4	41.6 ± 0.1	242.4 ± 13.1	16	41.6 ± 1.0	242.4 ± 15.9	0
	PND 17-21	Control	4	44.6 ± 1.4	249.3 ± 14.9	16	44.6 ± 2.2	249.3 ± 19.6	0
		10 mg/kg	4	44.1 ± 0.9	253.6 ± 6.1	16	44.1 ± 1.8	253.6 ± 13.4	0
		30 mg/kg	4	43.8 ± 0.9	257.7 ± 10.5	16	43.8 ± 1.8	257.7 ± 19.2	0
		100 mg/kg	4	42.6 ± 0.8	242.2 ± 14.6	16	42.6 ± 1.2**	242.2 ± 16.1	0
	PND 35-39	Control	4	43.6 ± 1.9	248.0 ± 4.7	16	43.6 ± 2.0	248.0 ± 14.2	0
		10 mg/kg	3	43.9 ± 0.8	256.4 ± 17.1	12	43.9 ± 1.4	256.4 ± 18.7	0
		30 mg/kg	3	46.8 ± 0.7*	267.4 ± 22.2	12	46.8 ± 1.4**	267.4 ± 29.0	0
		100 mg/kg	3	45.5 ± 1.1	261.5 ± 12.5	12	45.5 ± 1.7*	261.5 ± 17.9	0
DES	PND 1-5	Control	4	44.4 ± 1.9	259.5 ± 27.5	16	44.4 ± 2.5	259.5 ± 30.4	0
		10 μg/kg	4	44.9 ± 0.8	261.4 ± 25.1	16	44.9 ± 1.5	261.4 ± 27.5	0
		100 μg/kg	4	48.9 ± 3.4	311.8 ± 42.0	16	48.9 ± 4.0**	311.8 ± 46.0**	2 (2)
		300 μg/kg	4	49.9 ± 3.0*	288.0 ± 33.5	16	49.9 ± 3.9**	288.0 ± 39.8	3 (2)
	PND 17-21	Control	4	44.5 ± 1.3	242.7 ± 16.0	15	44.5 ± 2.1	241.2 ± 21.7	0
		10 μg/kg	3	46.3 ± 1.6	250.4 ± 7.2	12	46.3 ± 2.3	250.4 ± 19.9	0
		100 μg/kg	4	44.8 ± 0.9	243.3 ± 11.3	16	44.8 ± 1.1	243.3 ± 15.3	0
		300 μg/kg	4	45.4 ± 1.0	253.1 ± 12.7	16	45.4 ± 2.0	253.1 ± 22.2	0
	PND 35-39	Control	4	44.4 ± 1.9	251.6 ± 19.3	16	44.4 ± 2.2	251.6 ± 24.6	0
		10 μg/kg	3	43.9 ± 0.8	240.8 ± 32.7	12	43.9 ± 1.6	240.8 ± 32.5	0
		100 μg/kg	4	46.3 ± 1.4	243.3 ± 14.8	16	46.3 ± 2.4*	243.3 ± 19.8	0
		300 μg/kg	4	48.7 ± 2.2*	267.5 ± 34.1	16	48.7 ± 2.6**	267.5 ± 34.0	0
EE	PND 1-5	Control	4	43.3 ± 0.6	267.5 ± 4.8	16	43.3 ± 1.6	267.5 ± 17.4	0
		10 μg/kg	3	47.9 ± 4.3	282.7 ± 38.6	12	47.9 ± 4.6*	282.7 ± 39.3	1 (1)
		100 μg/kg	3	55.4 ± 1.0*	365.8 ± 9.1	12	55.4 ± 1.5**	365.8 ± 16.3**	10 (3)
	PND 6-10	Control	3	43.8 ± 1.1	245.7 ± 15.3	12	43.8 ± 1.8	245.7 ± 18.3	0
		10 μg/kg	4	48.4 ± 1.2**	287.3 ± 10.3**	16	48.4 ± 3.5**	287.3 ± 35.2**	1 (1)
	PND 11-15	Control	3	43.2 ± 0.9	257.0 ± 10.9	12	43.2 ± 1.2	257.0 ± 12.3	0
		10 μg/kg	4	44.1 ± 1.0	264.5 ± 14.1	16	44.1 ± 1.2	264.5 ± 19.3	0
	PND 17-21	Control	3	44.0 ± 0.8	243.7 ± 22.0	12	44.0 ± 1.1	243.7 ± 23.7	0
		10 μg/kg	2	43.8 ± 0.7	239.4 ± 20.9	8	43.8 ± 1.9	239.4 ± 18.8	0
		100 μg/kg	3	43.8 ± 0.8	252.7 ± 6.6	12	43.8 ± 1.7	252.7 ± 13.0	0
	PND 35-39	Control	4	44.3 ± 0.4	251.7 ± 16.9	16	44.3 ± 1.1	251.7 ± 22.5	0
		10 μg/kg	4	43.4 ± 1.5	240.0 ± 18.1	16	43.4 ± 1.9	240.0 ± 22.6	0
		100 μg/kg	4	45.9 ± 1.9	254.0 ± 24.2	16	45.9 ± 2.2	254.0 ± 28.3	0
TAM	PND 1-5	Control	4	43.3 ± 0.6	267.5 ± 4.8	16	43.3 ± 1.6	267.5 ± 17.4	0
		0.3 mg/kg	4	46.1 ± 4.5	276.8 ± 51.9	16	46.1 ± 4.6	276.8 ± 54.0	2 (1)
		1 mg/kg	4	49.8 ± 5.1	291.3 ± 47.9	16	49.8 ± 5.0**	291.3 ± 50.2	5 (2)
		3 mg/kg	4	55.8 ± 0.5**	323.0 ± 23.5	15	55.7 ± 1.0**	325.2 ± 29.6**	14 (4)
	PND 17-21	Control	4	44.6 ± 1.4	249.3 ± 14.9	16	44.6 ± 2.2	249.3 ± 19.6	0
		0.3 mg/kg	3	43.3 ± 1.3	244.4 ± 15.1	11	43.2 ± 2.0	243.3 ± 25.6	0
		1 mg/kg	4	43.9 ± 1.8	243.1 ± 3.8	15	43.7 ± 3.2	243.2 ± 27.3	0
		3 mg/kg	3	43.7 ± 2.1	246.5 ± 14.4	12	43.7 ± 2.3	246.5 ± 17.8	0
	PND 35-39	Control	4	43.6 ± 1.9	248.0 ± 4.7	16	43.6 ± 2.0	248.0 ± 14.2	0
		0.3 mg/kg	3	45.2 ± 0.8	246.0 ± 27.7	12	45.2 ± 2.1	246.0 ± 29.7	0
		1 mg/kg	3	44.5 ± 1.3	235.4 ± 13.9	12	44.5 ± 3.2	235.4 ± 26.3	0
		3 mg/kg	3	44.6 ± 1.3	231.6 ± 10.2	12	44.6 ± 1.6	231.6 ± 14.2	0

Value: Mean ± S.D. using the litter as the unit or using individual data.

PND: postnatal day; PPS: preputial separation; FLU: flutamide; DDE: *p,p'*-dichlorodiphenyldichloroethylene; VZ: vinclozolin; DES: diethylstilbestrol; EE: ethynylestradiol; TAM: tamoxifen; BW: body weight.

Incomplete separation: number of animals (litters) with incomplete separation on PND 56.

*: significantly different from control, $p<0.05$; **: significantly different from control, $p<0.01$.

Table 6. Relative Organ Weights and Body Weights of PND 56 Male Rats Postnatally Exposed to Chemicals (Using the Litter as the Unit)

Chemical	Dosing period	Group	Litters	Body weight (g)	Testes (mg/g)	Epididymides (mg/g)	Ventral prostate (mg/g)	Seminal Vesicles (mg/g)
FLU	PND 1-5	Control	4	384.1 ± 19.6	7.281 ± 0.714	1.400 ± 0.079	1.025 ± 0.137	2.008 ± 0.289
		1 mg/kg	4	378.1 ± 15.6	7.536 ± 0.457	1.511 ± 0.121	0.986 ± 0.175	1.895 ± 0.239
		10 mg/kg	4	389.8 ± 28.3	7.441 ± 0.713	1.443 ± 0.074	0.998 ± 0.112	1.774 ± 0.216
		30 mg/kg	4	371.4 ± 17.0	7.594 ± 0.583	1.438 ± 0.087	1.050 ± 0.099	1.806 ± 0.244
	PND 17-21	Control	3	376.2 ± 5.9	7.446 ± 0.481	1.438 ± 0.082	0.989 ± 0.051	1.758 ± 0.220
		1 mg/kg	3	358.8 ± 11.3	7.389 ± 0.075	1.489 ± 0.214	1.007 ± 0.093	1.835 ± 0.266
		10 mg/kg	3	372.1 ± 14.1	7.508 ± 0.469	1.451 ± 0.062	0.951 ± 0.188	1.918 ± 0.064
		30 mg/kg	3	376.4 ± 16.4	7.707 ± 0.172	1.484 ± 0.040	1.043 ± 0.041	1.923 ± 0.349
	PND 35-39	Control	4	377.3 ± 30.6	7.654 ± 0.302	1.520 ± 0.039	1.068 ± 0.076	2.065 ± 0.132
		1 mg/kg	4	382.8 ± 15.8	8.108 ± 0.773	1.603 ± 0.153	1.097 ± 0.112	2.108 ± 0.099
		10 mg/kg	4	378.5 ± 19.9	7.742 ± 0.725	1.407 ± 0.118	1.051 ± 0.121	1.774 ± 0.235
		30 mg/kg	4	381.1 ± 15.5	7.951 ± 0.378	1.398 ± 0.093	0.866 ± 0.106*	1.788 ± 0.131
DDE	PND 1-5	Control	3	362.5 ± 6.2	7.760 ± 0.192	1.447 ± 0.068	0.809 ± 0.084	1.942 ± 0.217
		10 mg/kg	3	382.2 ± 10.3	7.122 ± 0.178	1.395 ± 0.067	0.852 ± 0.113	1.874 ± 0.138
		30 mg/kg	3	367.2 ± 27.2	7.670 ± 0.261	1.443 ± 0.091	0.928 ± 0.074	1.893 ± 0.259
		100 mg/kg	3	369.8 ± 19.6	7.458 ± 0.358	1.421 ± 0.052	0.826 ± 0.020	1.792 ± 0.049
		300 mg/kg	3	374.0 ± 13.3	8.024 ± 0.356	1.398 ± 0.086	0.749 ± 0.011	1.783 ± 0.185
	PND 17-21	Control	3	358.6 ± 11.8	8.522 ± 0.979	1.539 ± 0.052	0.815 ± 0.136	1.724 ± 0.319
		10 mg/kg	3	377.0 ± 7.0	7.804 ± 0.504	1.465 ± 0.128	0.893 ± 0.006	1.820 ± 0.284
		30 mg/kg	3	359.7 ± 12.9	7.864 ± 0.469	1.497 ± 0.098	0.864 ± 0.058	1.674 ± 0.171
		100 mg/kg	3	362.8 ± 2.4	8.263 ± 0.261	1.502 ± 0.012	0.840 ± 0.059	1.751 ± 0.095
		300 mg/kg	4	361.9 ± 33.6	8.344 ± 0.668	1.423 ± 0.088	0.848 ± 0.127	1.550 ± 0.114
	PND 35-39	Control	3	333.5 ± 26.1	7.844 ± 0.454	1.520 ± 0.116	0.925 ± 0.205	1.834 ± 0.318
		10 mg/kg	3	356.0 ± 22.3	7.362 ± 0.622	1.323 ± 0.115	0.875 ± 0.017	1.831 ± 0.110
		30 mg/kg	3	347.2 ± 40.7	7.500 ± 0.215	1.376 ± 0.028	0.908 ± 0.067	1.807 ± 0.172
		100 mg/kg	3	353.5 ± 23.0	7.823 ± 0.247	1.409 ± 0.108	0.847 ± 0.065	1.523 ± 0.249
VZ	PND 1-5	Control	4	367.5 ± 20.6	7.990 ± 0.894	1.601 ± 0.142	1.049 ± 0.151	2.151 ± 0.180
		10 mg/kg	3	382.0 ± 32.7	7.392 ± 0.531	1.477 ± 0.047	1.006 ± 0.055	2.059 ± 0.083
		30 mg/kg	4	380.1 ± 9.5	7.986 ± 0.644	1.500 ± 0.133	0.910 ± 0.118	2.049 ± 0.093
		100 mg/kg	4	377.9 ± 17.6	7.373 ± 0.152	1.582 ± 0.109	1.030 ± 0.094	2.116 ± 0.082
	PND 17-21	Control	4	357.5 ± 26.3	7.791 ± 0.654	1.432 ± 0.058	0.967 ± 0.094	1.903 ± 0.300
		10 mg/kg	4	366.8 ± 9.9	7.981 ± 0.225	1.562 ± 0.080	0.987 ± 0.018	2.041 ± 0.239
		30 mg/kg	4	378.3 ± 17.4	7.629 ± 0.610	1.550 ± 0.147	0.891 ± 0.105	1.931 ± 0.237
		100 mg/kg	4	373.2 ± 25.5	8.066 ± 0.507	1.532 ± 0.137	1.018 ± 0.103	2.105 ± 0.135
	PND 35-39	Control	4	369.8 ± 20.8	7.711 ± 0.696	1.502 ± 0.053	0.895 ± 0.041	1.752 ± 0.115
		10 mg/kg	3	373.3 ± 39.0	7.420 ± 0.420	1.572 ± 0.114	0.910 ± 0.048	1.911 ± 0.081
		30 mg/kg	3	361.7 ± 32.0	8.125 ± 0.253	1.546 ± 0.018	0.982 ± 0.042	1.885 ± 0.053
		100 mg/kg	3	363.1 ± 5.5	7.719 ± 0.280	1.508 ± 0.088	0.941 ± 0.058	1.907 ± 0.083
DES	PND 1-5	Control	4	367.5 ± 25.0	8.089 ± 0.473	1.560 ± 0.151	1.064 ± 0.097	1.948 ± 0.141
		10 μg/kg	4	365.7 ± 24.8	7.296 ± 0.588	1.391 ± 0.100	0.917 ± 0.139	1.870 ± 0.364
		100 μg/kg	4	383.6 ± 13.7	8.277 ± 1.331	1.431 ± 0.121	0.769 ± 0.088**	1.668 ± 0.537
		300 μg/kg	4	343.6 ± 19.7	7.840 ± 1.413	1.585 ± 0.206	0.829 ± 0.076*	1.323 ± 0.086
	PND 17-21	Control	4	343.3 ± 32.2	7.753 ± 0.315	1.554 ± 0.155	0.925 ± 0.046	1.952 ± 0.302
		10 μg/kg	3	337.2 ± 20.4	7.906 ± 0.530	1.432 ± 0.075	0.922 ± 0.083	1.781 ± 0.050
		100 μg/kg	4	348.9 ± 19.6	6.940 ± 0.696	1.455 ± 0.105	0.900 ± 0.095	1.939 ± 0.281
		300 μg/kg	4	352.8 ± 13.8	7.309 ± 0.751	1.417 ± 0.093	0.933 ± 0.099	1.935 ± 0.317
	PND 35-39	Control	4	363.7 ± 38.2	7.750 ± 0.452	1.542 ± 0.103	1.052 ± 0.045	2.303 ± 0.295
		10 μg/kg	3	344.9 ± 40.5	7.472 ± 0.696	1.593 ± 0.146	1.035 ± 0.113	1.963 ± 0.221
		100 μg/kg	4	330.4 ± 23.8	7.920 ± 0.561	1.556 ± 0.024	0.987 ± 0.160	1.910 ± 0.240
		300 μg/kg	4	336.5 ± 36.0	7.986 ± 0.322	1.611 ± 0.100	0.970 ± 0.075	2.017 ± 0.201
EE	PND 1-5	Control	4	391.7 ± 14.7	7.908 ± 0.276	1.533 ± 0.097	1.024 ± 0.084	1.947 ± 0.140
		10 μg/kg	3	367.0 ± 22.8	7.681 ± 0.568	1.477 ± 0.094	0.850 ± 0.087	1.548 ± 0.136*
		100 μg/kg	3	374.0 ± 6.1	6.920 ± 0.110*	1.394 ± 0.112	0.700 ± 0.096**	1.220 ± 0.123**
	PND 17-21	Control	3	353.9 ± 32.7	7.154 ± 0.245	1.456 ± 0.062	0.908 ± 0.127	1.978 ± 0.041
		10 μg/kg	2	357.0 ± 27.3	7.201 ± 0.472	1.366 ± 0.099	1.013 ± 0.143	1.875 ± 0.179
		100 μg/kg	3	373.1 ± 21.3	6.980 ± 0.316	1.521 ± 0.049	1.053 ± 0.160	2.118 ± 0.156
	PND 35-39	Control	4	357.4 ± 26.0	7.766 ± 0.479	1.567 ± 0.098	1.078 ± 0.174	1.954 ± 0.102
		10 μg/kg	4	353.6 ± 9.4	7.586 ± 0.739	1.569 ± 0.131	0.960 ± 0.084	1.910 ± 0.236
		100 μg/kg	4	347.1 ± 27.6	7.637 ± 0.700	1.517 ± 0.119	0.936 ± 0.104	1.783 ± 0.262
TAM	PND 1-5	Control	4	391.7 ± 14.7	7.908 ± 0.276	1.533 ± 0.097	1.024 ± 0.084	1.947 ± 0.140
		0.3 mg/kg	4	368.7 ± 20.3	7.153 ± 0.691	1.498 ± 0.084	0.813 ± 0.120	1.625 ± 0.271
		1 mg/kg	4	356.4 ± 22.4	6.756 ± 1.014	1.454 ± 0.198	0.790 ± 0.128*	1.601 ± 0.242
		3 mg/kg	4	325.6 ± 25.8**	5.011 ± 0.351**	1.159 ± 0.042**	0.596 ± 0.112**	1.197 ± 0.257**
	PND 17-21	Control	4	357.5 ± 26.3	7.791 ± 0.654	1.432 ± 0.058	0.967 ± 0.094	1.903 ± 0.300
		0.3 mg/kg	3	365.3 ± 22.5	7.788 ± 0.213	1.524 ± 0.155	0.935 ± 0.178	1.937 ± 0.160
		1 mg/kg	4	351.9 ± 31.7	7.990 ± 0.766	1.541 ± 0.119	0.972 ± 0.264	1.871 ± 0.139
		3 mg/kg	3	363.5 ± 15.9	7.457 ± 0.510	1.516 ± 0.095	0.927 ± 0.131	2.060 ± 0.090
	PND 35-39	Control	4	369.8 ± 20.8	7.711 ± 0.696	1.502 ± 0.053	0.895 ± 0.041	1.752 ± 0.115
		0.3 mg/kg	3	348.8 ± 37.5	7.357 ± 0.352	1.548 ± 0.074	0.900 ± 0.075	1.733 ± 0.126
		1 mg/kg	3	348.8 ± 37.4	8.189 ± 0.358	1.581 ± 0.136	0.959 ± 0.079	1.909 ± 0.235
		3 mg/kg	3	339.8 ± 10.0	8.159 ± 0.212	1.626 ± 0.133	0.934 ± 0.119	1.866 ± 0.138

Value: Mean ± S.D. calculated using the litter as the unit.

FLU: flutamide; DDE: *p,p'*-dichlorodiphenyldichloroethylene; VZ: vinclozolin; DES: diethylstilbestrol; EE: ethynylestradiol; TAM: tamoxifen; PND: postnatal day.

*: significantly different from control, $p<0.05$; **: significantly different from control, $p<0.01$.

Table 7. Relative Organ Weights and Body Weights of PND 56 Male Rats Postnatally Exposed to Chemicals (Using the Individual Data)

Chemical	Dosing period	Group	Males	Body weight (g)	Testes (mg/g)	Epididymides (mg/g)	Ventral prostate (mg/g)	Seminal Vesicles (mg/g)
FLU	PND 1-5	Control	15	383.1 ± 24.2	7.294 ± 0.799	1.397 ± 0.105	1.014 ± 0.170	1.986 ± 0.304
		1 mg/kg	16	378.1 ± 21.8	7.536 ± 0.528	1.511 ± 0.130	0.986 ± 0.194	1.895 ± 0.255
		10 mg/kg	16	389.8 ± 34.8	7.441 ± 0.751	1.443 ± 0.111	0.998 ± 0.146	1.774 ± 0.249
		30 mg/kg	15	371.0 ± 27.8	7.574 ± 0.684	1.431 ± 0.107	1.049 ± 0.112	1.801 ± 0.276
	PND 17-21	Control	12	376.2 ± 15.8	7.446 ± 0.542	1.438 ± 0.124	0.989 ± 0.124	1.758 ± 0.292
		1 mg/kg	11	359.1 ± 21.3	7.388 ± 0.609	1.511 ± 0.205	1.016 ± 0.132	1.862 ± 0.307
		10 mg/kg	12	372.1 ± 27.8	7.508 ± 0.590	1.451 ± 0.084	0.951 ± 0.185	1.918 ± 0.170
		30 mg/kg	12	376.4 ± 20.4	7.707 ± 0.428	1.484 ± 0.100	1.043 ± 0.127	1.923 ± 0.388
	PND 35-39	Control	16	377.3 ± 33.2	7.654 ± 0.377	1.520 ± 0.067	1.068 ± 0.094	2.065 ± 0.238
		1 mg/kg	16	382.8 ± 26.3	8.108 ± 0.811	1.603 ± 0.165	1.097 ± 0.172	2.108 ± 0.301
		10 mg/kg	15	378.4 ± 26.7	7.778 ± 0.859	1.411 ± 0.139	1.053 ± 0.192	1.755 ± 0.324*
		30 mg/kg	16	381.1 ± 22.1	7.951 ± 0.487	1.398 ± 0.114*	0.866 ± 0.154**	1.788 ± 0.266*
DDE	PND 1-5	Control	12	362.5 ± 14.6	7.760 ± 0.369	1.447 ± 0.090	0.809 ± 0.130	1.942 ± 0.267
		10 mg/kg	12	382.2 ± 32.2	7.122 ± 0.498*	1.395 ± 0.108	0.852 ± 0.169	1.874 ± 0.226
		30 mg/kg	12	367.2 ± 26.8	7.670 ± 0.442	1.443 ± 0.102	0.928 ± 0.112	1.893 ± 0.292
		100 mg/kg	11	367.9 ± 28.3	7.460 ± 0.544	1.422 ± 0.074	0.826 ± 0.098	1.789 ± 0.214
		300 mg/kg	12	374.0 ± 26.9	8.024 ± 0.658	1.398 ± 0.096	0.749 ± 0.084	1.783 ± 0.288
	PND 17-21	Control	12	358.6 ± 24.0	8.522 ± 1.002	1.539 ± 0.142	0.815 ± 0.171	1.724 ± 0.334
		10 mg/kg	12	377.0 ± 25.9	7.804 ± 0.660	1.465 ± 0.131	0.893 ± 0.077	1.830 ± 0.308
		30 mg/kg	11	358.8 ± 24.7	7.830 ± 0.568	1.489 ± 0.109	0.865 ± 0.095	1.676 ± 0.174
		100 mg/kg	12	362.8 ± 18.3	8.263 ± 0.382	1.502 ± 0.064	0.840 ± 0.103	1.751 ± 0.211
		300 mg/kg	13	359.1 ± 38.0	8.731 ± 0.864	1.430 ± 0.124	0.841 ± 0.159	1.558 ± 0.178
	PND 35-39	Control	12	333.5 ± 26.1	7.844 ± 0.731	1.520 ± 0.124	0.925 ± 0.209	1.834 ± 0.331
		10 mg/kg	12	356.0 ± 24.1	7.362 ± 0.634	1.323 ± 0.112**	0.875 ± 0.084	1.831 ± 0.153
		30 mg/kg	12	347.2 ± 41.8	7.500 ± 0.372	1.376 ± 0.069*	0.908 ± 0.097	1.807 ± 0.238
		100 mg/kg	12	353.5 ± 26.9	7.823 ± 0.492	1.409 ± 0.156	0.847 ± 0.119	1.523 ± 0.247**
VZ	PND 1-5	Control	15	368.9 ± 30.7	7.917 ± 1.053	1.590 ± 0.158	1.039 ± 0.153	2.168 ± 0.277
		10 mg/kg	12	382.0 ± 35.0	7.392 ± 0.688	1.477 ± 0.100	1.006 ± 0.137	2.059 ± 0.160
		30 mg/kg	15	379.6 ± 16.8	8.020 ± 0.761	1.505 ± 0.136	0.917 ± 0.130	2.048 ± 0.302
		100 mg/kg	15	376.3 ± 24.8	7.361 ± 1.030	1.589 ± 0.134	1.025 ± 0.187	2.115 ± 0.195
	PND 17-21	Control	16	357.5 ± 28.1	7.791 ± 0.655	1.432 ± 0.086	0.967 ± 0.133	1.903 ± 0.317
		10 mg/kg	16	366.8 ± 24.6	7.981 ± 0.658	1.562 ± 0.126	0.987 ± 0.101	2.041 ± 0.302
		30 mg/kg	16	378.3 ± 26.0	7.629 ± 0.831	1.550 ± 0.182	0.891 ± 0.117	1.931 ± 0.287
		100 mg/kg	15	373.2 ± 30.3	8.056 ± 0.710	1.527 ± 0.146	1.022 ± 0.156	2.117 ± 0.277
	PND 35-39	Control	16	369.8 ± 25.8	7.711 ± 0.746	1.502 ± 0.098	0.895 ± 0.153	1.752 ± 0.190
		10 mg/kg	12	373.3 ± 35.7	7.420 ± 0.647	1.572 ± 0.122	0.910 ± 0.100	1.911 ± 0.231
		30 mg/kg	12	361.7 ± 31.7	8.125 ± 0.562	1.546 ± 0.076	0.982 ± 0.114	1.885 ± 0.154
		100 mg/kg	12	363.1 ± 14.2	7.719 ± 0.393	1.508 ± 0.134	0.941 ± 0.132	1.907 ± 0.203
DES	PND 1-5	Control	16	367.5 ± 31.8	8.089 ± 0.914	1.560 ± 0.174	1.064 ± 0.155	1.948 ± 0.262
		10 μg/kg	16	365.7 ± 26.6	7.296 ± 0.829	1.391 ± 0.152*	0.917 ± 0.167*	1.870 ± 0.390
		100 μg/kg	16	383.6 ± 23.0	8.277 ± 2.095	1.431 ± 0.127	0.769 ± 0.129**	1.668 ± 0.527
		300 μg/kg	15	343.0 ± 38.6	7.893 ± 2.723	1.569 ± 0.271	0.835 ± 0.120**	1.321 ± 0.232**
	PND 17-21	Control	15	340.4 ± 35.0	7.783 ± 0.497	1.566 ± 0.156	0.927 ± 0.097	1.962 ± 0.353
		10 μg/kg	12	337.2 ± 26.7	7.906 ± 0.674	1.432 ± 0.105*	0.922 ± 0.132	1.781 ± 0.209
		100 μg/kg	16	348.9 ± 24.8	6.940 ± 0.743**	1.455 ± 0.118	0.900 ± 0.109	1.939 ± 0.283
		300 μg/kg	16	352.8 ± 20.5	7.309 ± 0.846	1.417 ± 0.121**	0.933 ± 0.117	1.935 ± 0.346
	PND 35-39	Control	15	360.3 ± 36.2	7.793 ± 0.609	1.539 ± 0.168	1.052 ± 0.194	2.299 ± 0.488
		10 μg/kg	12	344.9 ± 39.7	7.472 ± 0.686	1.593 ± 0.155	1.035 ± 0.140	1.963 ± 0.240
		100 μg/kg	16	330.4 ± 26.3	7.920 ± 0.736	1.556 ± 0.089	0.987 ± 0.193	1.910 ± 0.351
		300 μg/kg	16	336.5 ± 36.5	7.986 ± 0.777	1.611 ± 0.153	0.970 ± 0.134	2.017 ± 0.260
EE	PND 1-5	Control	16	391.7 ± 17.3	7.908 ± 0.437	1.533 ± 0.131	1.024 ± 0.128	1.947 ± 0.269
		10 μg/kg	12	367.0 ± 22.9**	7.681 ± 0.690	1.477 ± 0.103	0.850 ± 0.110**	1.548 ± 0.270**
		100 μg/kg	10	372.7 ± 15.4*	6.919 ± 0.402**	1.387 ± 0.149*	0.692 ± 0.114**	1.223 ± 0.166**
	PND 17-21	Control	12	353.9 ± 32.3	7.154 ± 0.561	1.456 ± 0.126	0.908 ± 0.147	1.978 ± 0.207
		10 μg/kg	8	357.0 ± 27.4	7.201 ± 0.538	1.366 ± 0.101	1.013 ± 0.149	1.875 ± 0.277
		100 μg/kg	12	373.1 ± 23.1	6.980 ± 0.530	1.521 ± 0.101	1.053 ± 0.187	2.118 ± 0.245
	PND 35-39	Control	16	357.4 ± 29.4	7.766 ± 0.783	1.567 ± 0.118	1.078 ± 0.196	1.954 ± 0.286
		10 μg/kg	16	353.6 ± 17.8	7.586 ± 0.738	1.569 ± 0.149	0.960 ± 0.147	1.910 ± 0.347
		100 μg/kg	16	347.1 ± 31.1	7.637 ± 0.870	1.517 ± 0.144	0.936 ± 0.123 *	1.783 ± 0.267
TAM	PND 1-5	Control	16	391.7 ± 17.3	7.908 ± 0.437	1.533 ± 0.131	1.024 ± 0.128	1.947 ± 0.269
		0.3 mg/kg	16	368.7 ± 27.9*	7.153 ± 0.772	1.498 ± 0.115	0.813 ± 0.161**	1.625 ± 0.352*
		1 mg/kg	16	356.4 ± 28.8**	6.756 ± 1.135*	1.454 ± 0.219	0.790 ± 0.181**	1.601 ± 0.403*
		3 mg/kg	15	327.9 ± 29.0**	5.043 ± 0.550**	1.162 ± 0.114**	0.606 ± 0.124**	1.218 ± 0.371**
	PND 17-21	Control	16	357.5 ± 28.1	7.791 ± 0.655	1.432 ± 0.086	0.967 ± 0.133	1.903 ± 0.317
		0.3 mg/kg	11	364.5 ± 29.6	7.768 ± 0.452	1.530 ± 0.189	0.950 ± 0.196	1.936 ± 0.244
		1 mg/kg	15	354.0 ± 31.8	7.913 ± 0.812	1.530 ± 0.126	0.945 ± 0.243	1.873 ± 0.236
		3 mg/kg	12	363.5 ± 21.1	7.457 ± 0.901	1.516 ± 0.121	0.927 ± 0.212	2.060 ± 0.374
	PND 35-39	Control	16	369.8 ± 25.8	7.711 ± 0.746	1.502 ± 0.098	0.895 ± 0.153	1.752 ± 0.190
		0.3 mg/kg	12	348.8 ± 37.5	7.357 ± 0.457	1.548 ± 0.095	0.900 ± 0.102	1.733 ± 0.173
		1 mg/kg	12	348.8 ± 42.4	8.189 ± 0.787	1.581 ± 0.134	0.959 ± 0.141	1.909 ± 0.291
		3 mg/kg	12	339.8 ± 16.1*	8.159 ± 0.596	1.626 ± 0.134*	0.934 ± 0.139	1.866 ± 0.194

Value: Mean ± S.D. calculated using the individual data.

FLU: flutamide; DDE: *p,p'*-dichlorodiphenyldichloroethylene; VZ: vinclozolin; DES: diethylstilbestrol; EE: ethynylestradiol; TAM: tamoxifen; PND: postnatal day; *: significantly different from control, $p<0.05$; **: significantly different from control, $p<0.01$.

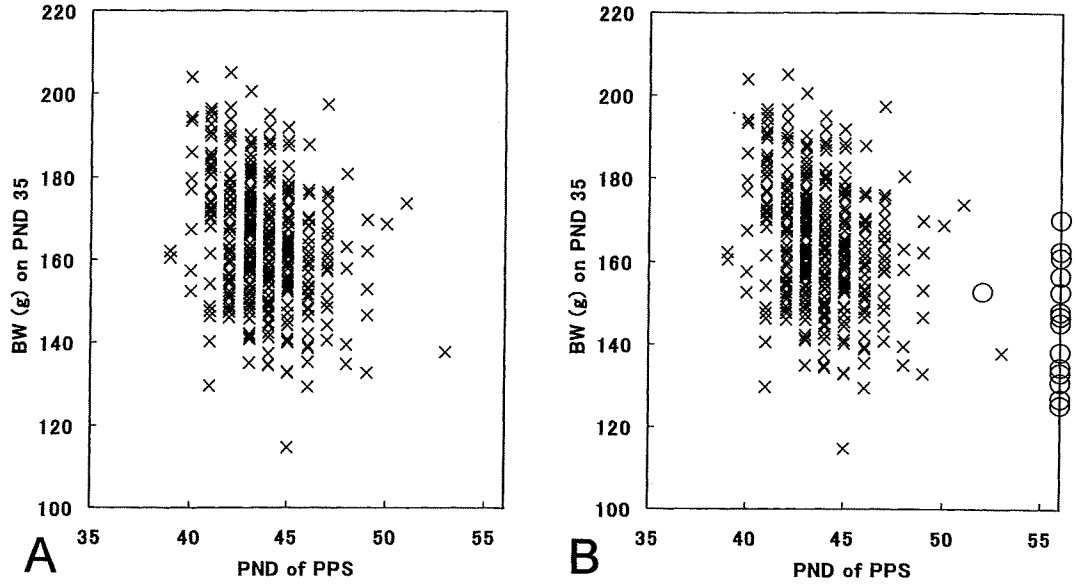

Fig. 5. Correlation diagram between the day of preputial separation and body weight of control and dosed rats. A: There is a negative correlation between PND of preputial separation (PPS) and body weight on PND 35. Number of cases = 366, Pearson's correlation coefficient: r = –0.30. B: Preputial separation (PPS) of males exposed to 3 mg/kg tamoxifen on PND 1–5 is delayed and the data are plotted as "O" outside the range of control data "X".

3) Pathological examination

Unilateral or bilateral cloudy white discoloration of the efferent ductule as well as testis enlargement was increased in the groups exposed to EE and DES on PND 1–5. Histopathological examination revealed retention of sperm or inflammatory cells in the lumen of the efferent ductule and edema in adipose tissue surrounding the dilated ductule. Organs of the EE or TAM groups showed no remarkable histological changes except for a relative atrophic appearance.

Correlation between preputial separation and body weight

The control animal data in this study (n=366) are symbolized by "X" on the scattergram of Fig. 5A. PND of preputial separation and body weight on PND 35 showed a negative correlation (Pearson's correlation coefficient: r = –0.30). The data from animals treated with 3 mg/kg TAM, symbolized by "O", are compared in Fig. 5B. Body weight on PND 35 in this group showed significant reduction. Preputial separation of TAM treated males was delayed, and the data were outside the range of the control data.

Discussion

Preputial separation in untreated rats initiates from cornification of the epithelium lying between the glans penis and prepuce[1,2]. The cornification progresses from the tip of the glans penis towards its base and from the dorsal surface to ventral aspect of the glans penis. Preputial separation is considered complete when cornification reaches the ventral end of the glans penis. However, complete separation was not observed in animals exposed to FLU or VZ in their fetal period, since they had a cleft phallus and hypospadias. Induction of hypospadias is reportedly caused by FLU, VZ and finasteride[9,14–19]. FLU, a well-known potent androgen receptor antagonist, is used as a non-steroidal, anti-androgen drug for the treatment of prostate cancer. FLU inhibits TS and DHT binding to the intracellular androgen receptor, and prenatal/perinatal FLU exposure induces abnormalities in the genital tract of rats such as hypospadias and agenesis of the prostate, epididymis, and vas deferens[9,14,15]. The fungicide, VZ, is also an androgen receptor antagonist. It induces hypospadias in rats after perinatal or prenatal administration[16,17]. Finasteride, which inhibits 5α-reductase conversion of TS to DHT, also induces hypospadias in male rats exposed to it from GD 15 to day 21 postpartum[18] or GD 6–20[19], and based on this finding, DHT is thought to be involved in the development of the external genitalia.

The most sensitive period to induce hypospadias is reportedly GD 15–16 with 400 mg/kg of VZ[17], while only weak sensitivity was found with treatment on GD 17–18. Finasteride-exposed rats also showed similar results[18]. In our present study, exposure to 100 mg/kg of VZ on GD 14–17 induced hypospadias and a cleft phallus with cleft prepuce, but exposure on GD 18–21 did not induce any abnormalities in the external genitalia. At a higher dose of 200 mg/kg, VZ caused the death of the pregnant females or newborn pups (data not shown). Exposure to 10 mg/kg of FLU on GD 14–17 induced hypospadias with a cleft phallus and cleft prepuce, while exposure on GD 18–21 induced hypospadias and a cleft phallus without a cleft prepuce.

Higher doses of FLU induced the same abnormality as our previous study[2]. Although both FLU and VZ are androgen receptor antagonists, their sensitive periods differed: cleft phallus was caused by FLU administration until later in pregnancy. FLU exposed males without the malformation showed a hypoplastic penis and a delay in preputial separation, but prenatal exposure to other chemicals did not affect the preputial separation.

Postnatal chemical exposure influenced preputial separation in a variety of ways. Anti-androgen, FLU caused a delay when administered on PND 35–39, and statistical analyses using individual data of DDE and VZ also showed significant delays of preputial separation, but neonatal exposure to these chemicals on PND 1–5 did not influence the time of preputial separation. On the other hand, neonatal administration of EE and TAM induced a marked delay or incomplete separation. DES exposure during both PND 1–5 and PND 35–39 caused a delay in separation. Preputial separation is thought to be dependent on the continued presence of androgen after PND 35, since castration on PND 35 blocks preputial separation and the addition of TS or DHT reverses the effects of castration[3]. Male rat serum testosterone levels have been reported to decrease after birth and to increase at the prepubertal stage[11]. Testosterone during fetal life is thought to act to masculinize the genitalia, and testosterone at puberty may act on the maturation of the target organ. Prenatal exposure to the anti-androgens used in the present study resulted in malformation of the external genitalia, and the effect of their prepubertal exposure was a delay in preputial separation. These results may indicate that anti-androgens effect on male rats in a relatively higher level of serum testosterone, and that male rats are not sensitive to neonatal exposure to anti-androgenic chemicals.

Neonatal exposure of estrogenic chemicals is known to induce marked effects on male rats. Delay of preputial separation and decrease of testis and prostate weight with a reduction of plasma testosterone levels have been reported in rats neonatally administered estradiol benzoate[20]. Neonatal treatment of DES or EE has also been reported to cause dose-dependent reductions in plasma testosterone levels and testis weights in adulthood[21]. An estrogen receptor is found in the male reproductive tract[22,23]. These findings suggest that estrogen is relevant to the growth of the male reproductive organs. The delay of preputial separation induced by DES, EE and TAM exposures on PND 1–5 in the present study is thought to be caused by estrogen-related effects, and the delay may have not only been caused by the direct effects on the genital tract but also by effects on the systemic endocrine function. Prepubertal exposure to DES induced a delay of preputial separation in male rats in the present study, the same as observed for anti-androgenic chemicals. Serum testosterone levels in adult or prepubertal male rats have been reported to be increased by FLU[24,25], DDE[5] and VZ[24] treatment, and the increase is thought to be caused by their anti-androgenic effects. It has been reported that serum testosterone levels in adult male rats treated with DES are decreased[26], thus, testosterone reduction may be the reason for the delay of preputial separation seen in the DES group prepubertally treated in our present study. EE and TAM have also been reported to decrease serum testosterone levels[25,27], but these chemicals did not delay preputial separation in our present study. The reason for the different effects of DES and EE/TAM treatments were not revealed in our study.

A negative correlation between the body weight on PND 35 and the day of preputial separation was demonstrated in the control males (Fig. 5A). This diagram shows a tendency for males of higher body weight to complete preputial separation earlier than males of lower body weight. Ashby and Lefevre[8] thought that there was a marked dependence of the day of preputial separation on the initial body weight of the test animals, and that delays in preputial separation can only be interpreted with confidence when they are not accompanied by losses in body weight. In our present study, males exposed to 3 mg/kg TAM on PND 1–5 showed significantly lower body weight on PND 35, and their preputial separation was delayed. The delay may not depend on the reduced weight gain, since 14 males out of 15 did not show complete separation of the prepuce on the day of autopsy, PND 56, and their data were outside the range of control data.

AGD was reduced by FLU and VZ exposures on GD 14–17. Reduction of AGD by anti-androgen has been reported, and AGD is thought to have high sensitivity to anti-andorogens[17]. In the present study, however, there were no apparent changes in AGD after DDE exposure. DES also caused a decrease in AGD with GD 18–21 exposure, and there was a difference between the results of DES and EE/TAM exposure. Histological examination revealed a tortuous and bent cavernous body of the penis seen in the sagittal section of males prenatally exposed to FLU or VZ and sacrificed on PND 6. This morphological change may be a reason for the reduction in AGD.

The relative weights of the ventral prostate in males prenatally exposed to VZ or DES were decreased significantly. In these groups, prostate aplasia or hypoplasia was observed in the group exposed to VZ on GD 14–17 and DES on GD 18–21. Postnatal FLU exposure decreased the relative weight of the ventral prostate in the group dosed on PND 35–39. No apparent effect was detected in males postnatally exposed to DDE or VZ. PND 1–5 exposure of EE or TAM induced a reduction in the weight of the testis and other reproductive organs. Although the statistical analyses using the individual data showed significant results in the lower dose group and the other organs, there were no significant differences in the groups exposed to VZ. These findings indicate that preputial separation is more useful than the measurement of organ weight as an endpoint in detecting endocrine active chemicals under the conditions used in this study.

Prenatal exposure of anti-androgens FLU and VZ induced hypospadias, and the time of preputial separation could not be determined. Although GD 18–21 exposure was expected to produce a very low incidence of hypospadias,

FLU exposure on GD 18–21 induced the abnormality. Other chemicals did not induce hypospadias or delays of the preputial separation. From these results, it is unclear if preputial separation after prenatal exposure is a useful way for detecting endocrine active chemicals. On the other hand, prepubertal exposure to FLU, DDE and VZ caused delays in preputial separation, and neonatal exposure to EE and TAM induced delays in separation with a reduction in organ and body weight gain. Both neonatal and prepubertal exposure to DES caused a delay in preputial separation. These results indicate that neonatal (PND 1–10) and prepubertal exposure may be useful for detecting endocrine active chemicals by observing preputial separation, and that continuous administration of chemicals from PND 35 to the day of preputial separation may be more effective for prepubertal exposure.

In conclusion, the usability of preputial separation to detect endocrine active chemicals after prenatal exposure to them is still unclear. Postnatal exposure, however, may be a useful method for a screening assay to detect endocrine active chemicals by preputial separation, and postnatal exposure is dependent on both neonatal (PND 1–10) and prepubertal continuous exposure.

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